The Biosynthesis of C^{14}-Labeled Lipids by Isolated Bull Spermatozoa*

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Washed bull spermatozoa were resuspended in phosphate saline and incubated with C^{14}-labeled substrates. The lipids were extracted and fractionated by chromatographic techniques. On incubation of the spermatozoa with glycerol-1-C^{14} under aerobic conditions, or with glucose-C^{14} (randomly labeled) under either aerobic or anaerobic conditions, diglycerides and phosphatides became labeled with C^{14} (specific activities 2000–4000 and 200 cpm/mg respectively). All the radioactivity of these fractions resided in their glycerol moiety. Incubation with acetate-1-C^{14} resulted in labeling of the fatty acids of those fractions, although only to a small extent. The results provide evidence for the existence of biosynthetic mechanisms in mature ejaculated bull spermatozoa. The diglyceride reserves of bull spermatozoa appear to be adequate to support their "endogenous" respiration by the oxidation of the fatty acids. Four ketosteroids and one C_{21}-steroid were present in the lipid extracts.

The composition of the lipids of the spermatozoa of the ram and bull has been studied in detail by Lovern *et al.* (1957). Lipids have long been implicated as a possible endogenous substrate in mammalian spermatozoa (Lardy and Phillips, 1943, 1945). Recently, Hartree and Mann (1959, 1960) suggested that the fatty acids of the acetal phosphatides of ram and bull spermatozoa provide the substrate for respiration in the absence of external carbohydrate. Although there can be no doubt that the lipids as well as other tissue components are formed at an early stage in the life cycle of the germ cell, there has been no conclusive evidence concerning the ability of mature spermatozoa to synthesize any of their constituents. Indeed, the prevailing view has appeared to be that the mature spermatozoon possesses only a catabolic mechanism for the production of energy for motility (see Terner, 1962).

The present study was undertaken to examine the question whether mature spermatozoa retain any biosynthetic capacity after ejaculation. The principal observation was that, on incubation with C^{14}-labeled glycerol or glucose, the glycerol-containing lipid fractions of washed bull spermatozoa became intensely labeled in the glycerol moiety under both aerobic and anaerobic conditions. Preliminary reports have been presented (Terner and Korsh, 1960; Terner, 1962).

MATERIALS AND METHODS

Freshly collected bull semen from one or several bulls was combined (total volume 10–20 ml) and centrifuged in an International clinical centrifuge for 20 minutes at 2000 rpm. The seminal plasma was removed and the cells were resuspended in phosphate saline without calcium, *p*H 7.4 (Krebs and Eggleston, 1940), and centrifuged for a further 15 minutes. The cells were finally suspended in a volume of phosphate saline approximately equal to the original volume of the whole semen. Twenty-ml portions were pipetted into 125-ml Erlenmeyer flasks. C^{14}-labeled substrate (100 μc/mmole) was added to give a final concentration of $1.25–2.5 \times 10^{-3}$ M. In aerobic incubations the gas phase was air. When anaerobic conditions were to be maintained, the flask containing the sperm suspension was gassed with 100% nitrogen and a short test tube containing a stick of yellow phosphorus was introduced. Gassing was continued through two hypodermic needles inserted through the serologic rubber cap closing the flask. Finally the radioactive substrate was injected through the cap and the flask was placed in a shaking water bath at 37°. After 120 minutes of incubation the flasks were chilled in cracked ice and the sperm cells were sedimented by centrifuging at 4°; they were resuspended and centrifuged twice in cold saline containing a small amount of unlabeled substrate. The moist mass of packed cells was then extracted by homogenization in a mixture of chloroform-methanol (1:1 v/v) followed by centrifugation. The extraction was repeated until the remaining tissue was practically free from lipid.

The crude lipid extract was washed according to the method of Folch *et al.* (1957). In order to remove all traces of radioactive contaminants, the washing had to be repeated seven or eight times, with addition of unlabeled substrate as carrier during the earlier stages, until the aqueous washes were free from radioactivity. In order to minimize losses due to the exhaustive washing process the combined aqueous methanolic washes were taken to dryness and the residue was redissolved in ethyl acetate. This solution was again washed with water until the washes were free from radioactivity. The lipid material recovered was added to the chloroform extract. The solution of lipid was

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taken to dryness under nitrogen, and the material was stored in a vacuum desiccator in the cold until sufficient material had been accumulated. The lipid extracts from 10–20 identical incubations were combined. On the average, 100 ml of fresh semen yielded 10^{11} washed spermatozoa, which contained *ca.* 100 mg of lipid and *ca.* 1 g of lipid-free dry residue.

Solvent Partition and Column Chromatography.—Since the lipid extract did not completely dissolve in hexane at room temperature, it was dissolved in hexane and methanol and partitioned in separating funnels between hexane and 90% methanol with seven transfers. Both the hexane (fraction A) and the aqueous methanol (fraction B) phases were taken to dryness under nitrogen. Fraction A was taken up in hexane and chromatographed on a silicic acid column according to the scheme of Mead and Fillerup (1954). Fraction B was dissolved in benzene, applied to a silica gel column, and eluted stepwise with benzene and ethyl acetate mixtures with increasing amounts of ethyl acetate (Levy *et al.*, 1954).

Separation of Glycerides, Cholesterol, and Steroids.—Preliminary work had shown that the radioactive fractions of highest specific activity were those eluted by a mixture of ethyl ether and hexane (1 : 3 and 1 : 1 v/v) in the course of the silicic acid column chromatography of fraction A and by benzene–ethyl acetate (1 : 1 v/v) on chromatography of fraction B on a silica gel column. These fractions gave a positive Liebermann–Burchard reaction and also showed the presence of ester groupings by the alkaline hydroxylamine test (Hestrin, 1949). Separation of the individual components was attempted by the following procedure, to which the fractions were subjected at first separately, in later experiments after combination. The material was chromatographed on paper in the heptane-propylene glycol system of Burton *et al.* (1951). After 16 hours at 24° the bulk of the material had run off the paper. Inspection of the paper in ultraviolet light revealed several zones which were also found to give a faint reaction with the Zimmermann reagent. The overrun was taken to dryness in a vacuum desiccator to remove all traces of solvent. It was then dissolved in a mixture of ethanol and acetone (1 : 1 v/v) and treated with a solution of 2% digitonin in 80% (v/v) ethanol. The digitonin-insoluble material was repeatedly extracted with fresh ethanol-acetone mixture and then with ethyl ether. The combined supernatant and washings were taken to dryness and the residue was exhaustively extracted with ethyl ether. The ether-soluble material, which contained almost all the radioactivity of the crude fractions, was rechromatographed on a silicic acid column with use of the sequence of solvent mixtures recommended by Barron and Hanahan (1958). The column retained most of the colored impurities and permitted the elution of a fraction of high specific activity which emerged with the solvent mixture containing 30% ethyl ether–70% hexane. The presence of ester bonds was shown by the alkaline hydroxylamine reaction. This suggested that the material was diglyceride. Attempts to purify the material by crystallization from cold methanol resulted in only a small increase in specific activity. Infrared spectra (melts on a NaCl crystal) showed the bands of ester carbonyl and hydroxyl groups and the progression bands characteristic of long-chain fatty acids (Bellamy, 1958). The material was further tested by chromatography on silicone-impregnated paper (Schlenk *et al.*, 1957) and on silicic acid–impregnated paper (Marinetti and Stotz, 1960). Both methods were useful in distinguishing diglycerides from triglycerides and cholesterol esters. The purified glycerides were transesterified by refluxing in methanol containing 1% sulfuric acid. The methyl esters of the fatty acids were extracted with hexane and analyzed by gas chromatography (Aerograph; 6-ft. column of Craig polyester succinate on chromosorb). The aqueous residue remaining after the extraction of the methyl esters was deionized by passage through an ion exchange column and concentrated by lyophilization. Portions of the aqueous concentrate were chromatographed on paper (Hanahan and Olley, 1958). Glycerol was detected by the periodate-fuchsin reaction (Geldmacher-Mallinckrodt and May, 1957) and, after elution from the paper, by the chromotropic acid reaction (Hanahan and Olley, 1958).

Identification of Cholesterol.—The digitonin-insoluble precipitate was cleaved by heating with pyridine (Bergmann, 1940). The regenerated sterol fraction was further purified by chromatography on aluminum oxide (Merck), followed by bromination and regeneration (Schwenk and Werthessen, 1952).

Ketosteroids.—The weakly Zimmermann-positive zones remaining on the paper after chromatography in the Zaffaroni system (Burton, Zaffaroni, and Keutmann, 1951) were eluted and rechromatographed on paper in a Bush (1952) system (hexane saturated with 90% aqueous methanol). Since the zones recovered from the individual batches were still faint and uncertain it was deemed necessary, in order to ensure sufficient material for purification and analysis, to combine the corresponding zones from all available batches irrespective of the radioactive substrate with which the spermatozoa had been incubated. After combination each zone was rechromatographed twice in the Bush system. The resulting zones were clearly defined by absorption of ultraviolet light on the paper strips and by the Zimmermann reaction. Finally the substances were eluted from the paper, crystallized from methanol and hexane, and dispersed in KBr for infrared analysis (Rosenkrantz *et al.*, 1958).

C_{21}-Steroids.—The material recovered from fraction B, which was eluted by 100% ethyl acetate, was run on paper in the system of Zaffaroni (1953) (toluene saturated with propylene glycol) followed by rechromatography in solvent system C of Bush

(1952). Reduction of blue tetrazolium was used for the detection of the material on the paper strips.

Phosphatides.—The fractions eluted from silicic acid columns by mixtures of methanol and ethyl ether were recombined, stored in absolute methanol at −17°, and then rechromatographed on silicic acid columns according to the scheme of Hanahan *et al.* (1957). The individual fractions were chromatographed on silicic acid–impregnated paper, with the solvent system diisobutyl ketone–acetic acid–water (40 : 30 : 7) (Marinetti and Stotz, 1956), 3 mg of material being applied to the paper strips along a 4.5-cm line. The chromatograms were developed for 20 hours. The lipid zones were localized by staining with Rhodamine 6G. Pigmented zones and areas showing fluorescence in ultraviolet light were discarded. The "clean" zones were eluted, rechromatographed on silicic acid–impregnated paper, and again localized by staining with Rhodamine 6G. Choline-containing phosphatides were tested for on guide strips by the phosphomolybdic-$SnCl_2$ reaction (Chargaff *et al.*, 1948), and amino groups by the ninhydrin reaction. After elution of the zones, crystallization was attempted with methanol or methanol and acetone used as solvents. After the determination of its radioactivity the crystalline material was dissolved in CS_2 and analyzed by infrared spectrophotometry.

Determination of Radioactivity.—Portions of all lipid fractions eluted from columns were plated on planchets in thin layers and counted in a gas-flow counter with a thin window. Radioactive zones on paper strips were detected by scanning in a chromatogram strip counter or by radioautography. When run on a preparative scale the zones were subsequently eluted from the paper, purified on a small silicic acid column and/or by crystallization, and counted on planchets in the gas flow counter. Specific activities were calculated as cpm/mg material at infinite thinness.

Results

Incorporation of C^{14} into Lipids of Spermatozoa.—As shown in Table I, incubation of spermatozoa with C^{14}-labeled glycerol or glucose resulted in the formation of radioactive lipids. Acetate-1-C^{14} was incorporated to a much lesser extent, even when supplemented with nonradioactive glycerol. No radioactivity appeared in the lipids of spermatozoa which had been immobilized by the addition of formaldehyde prior to incubation with the radioactive substrates.

Distribution of C^{14} in Sperm Lipid Fractions.—Table II shows a typical pattern of distribution of radioactive carbon in the various fractions of the lipids of spermatozoa which had been incubated with glycerol-1-C^{14} under aerobic conditions. A similar pattern resulted from incubation with randomly labeled glucose-C^{14} under either aerobic or anaerobic conditions. The fractions of highest specific activity were eluted from column A

Table I

C^{14}-Labeling of Lipids of Bull Spermatozoa *in Vitro*

Washed bull spermatozoa were incubated in phosphate saline at 37°. Under aerobic conditions, the gas was air; under anaerobic conditions, 100% nitrogen. Glucose or glycerol, 1.25×10^{-3} M; acetate, 2.5×10^{-3} M. The radioactive substrates added had a specific activity of 100 μc/mmole. After 2 hours' incubation, the lipids were extracted and dialyzed (Folch *et al.*, 1957). The lipid extract was partitioned between hexane and 90% aqueous methanol, yielding fractions A and B respectively.

Substrate	Conditions	Specific Activity of Lipid Fraction (cpm/mg) Folch Extract	A	B
Glycerol-1-C^{14}	Aerobic	176	422	86
Acetate-1-C^{14}	Aerobic	11	10	25
Glycerol-1-C^{14} + acetate-1-C^{14}	Aerobic	460	800	150
Glycerol-1-C^{14} + acetate	Aerobic	520	1340	208
Acetate-1-C^{14} + glycerol	Aerobic	22	26	22
Glucose-C^{14} (randomly labeled)	Aerobic	249	508	115
Glucose-C^{14} (randomly labeled)	Anaerobic	200	350	90
Glycerol-1-C^{14} + acetate-1-C^{14}	Aerobic ("killed")[a]	2		

[a] The spermatozoa were "killed" by the addition, prior to incubation with the radioactive substrates, of a few drops of formaldehyde until motility was abolished.

in fractions 4 and 5 and from column B in fraction 3. The material of fraction A-4 was shown to contain cholesterol by the Liebermann-Burchard reaction. An attempt at crystallization from methanol resulted in the formation of crystals of low specific activity. Treatment of fraction A-4 with digitonin resulted in the formation of a digitonide which on regeneration and further purification yielded cholesterol free from measurable radioactivity. After further purification of the "digitonin-soluble" portion by paper chromatography (Burton *et al.*, 1951) and silicic acid column chromatography (Barron and Hanahan, 1958), the final product had a specific activity of 3730 cpm/mg which was not further increased by crystallization from cold methanol. Fraction A-5 did not contain cholesterol; its specific activity after purification by the same methods as applied to A-4 increased to 3520 cpm/mg. Both substances melted at 60–61° and were identified as diglycerides as described in the section on Methods. Transesterification of the diglycerides yielded unlabeled methyl esters of fatty acids which on gas chromatographic analysis showed the peaks of myristic, palmitic, and stearic acids in approximately equal amounts, small amounts of lauric and oleic acids, and in some samples a trace of myristoleic and linoleic acids. The glycerol moiety recovered from the aqueous residue of the transesterification mixture contained all the radioactivity of the original diglycerides.

From the amounts of material in the crude fractions and the specific activities of the crude and purified products of the experiment shown in Table II it was calculated that fraction A-4 contained 23 mg cholesterol and 14.4 mg diglyceride. There

TABLE II

C^{14}-LABELING OF LIPID FRACTIONS OF ISOLATED BULL SPERMATOZOA

Washed spermatozoa incubated in phosphate saline at 37°; gas, air. Glycerol-1-C^{14}, 1.25×10^{-3} M; acetate-1-C^{14}, 2.5×10^{-3} M. Incubation period, 120 minutes. The spermatozoa were isolated from a total volume of 232 ml of fresh semen supplied in 13 portions over a period of 3 months. The total sperm count of the 13 washed suspensions as incubated was 31.83×10^{10}. Total lipid-free dry weight of spermatozoa 3.58 g. The crude lipid extract (377 mg; specific activity 460 cpm/mg) on partitioning between hexane and 90% aqueous methanol yielded fraction A (158.4 mg; specific activity 800 cpm/mg) and fraction B (183.6 mg; specific activity 150 cpm/mg). Column A: silicic acid; column B: silica gel. Each fraction was eluted by 250-ml portions of solvent mixtures.

Column	Fraction No.	Solvent	Wt. of Fraction (mg)	Specific Activity (cpm/mg)
A	1	Hexane	44.0	54
	2	Hexane–3% ethyl ether	7.1	10
	3	Hexane–10% ethyl ether	8.4	24
	4	Hexane–25% ethyl ether	37.4	1420
	5	Hexane–ethyl ether (1:1 v/v)	12.4	3250
	6	Ethyl ether	15.7	176
	7	Methanol–ethyl ether (1:3 v/v)	10.8	153
	8	Methanol–ethyl ether (1:1 v/v)	10.6	40
	9	Methanol–chloroform (1:1 v/v)	7.2	106
	10	Methanol	2.7	84
B	1	Benzene	152.1	171
	2	Benzene–ethyl acetate (9:1 v/v)	7.6	15
	3	Benzene–ethyl acetate (1:1 v/v)	7.6	560
	4	Ethyl acetate	2.6	50
	5	Ethyl acetate–methanol (1:1 v/v)	19.3	94

were also 12 mg diglyceride in fraction A-5 and 7 mg cholesterol in fraction B-3, so that the sperm lipids contained 6.9% diglyceride and 8% cholesterol. The lipids also include 8–10% of material eluted by solvent mixtures containing methanol and therefore assumed to be phosphatides.

C^{14}-Labeling of Sperm Phosphatides.—As shown in Table II, the phosphatide fractions of bull spermatozoa that had been incubated with C^{14}-labeled glycerol or glucose were radioactive. Chromatography on silicic acid–impregnated paper of the material eluted from silicic acid columns by mixtures of chloroform and methanol, 3 : 2 and 1 : 4 (v/v), yielded zones which contained choline. The zones were eluted from the paper and crystallized from cold methanol and acetone. The specific activity of the principal crystalline material was 226 cpm/mg. Its infrared spectrum in CS_2 solution showed the bands described by Freeman *et al.* (1957) as criteria for the identification of lecithin and corresponded to the infrared spectrum of a synthetic sample of lecithin.[1] Other radioactive zones containing choline found on the paper had lower R_F values and may have been lysophosphatides formed by degradation during paper chromatography or during the preceding storage. Their specific activities ranged from 100–200 cpm/mg. No attempt was made to hydrolyze the phosphatides isolated because of the small amounts available after completion of the isolation and purification procedures designed to ensure a high degree of radiopurity of the material. In view of the necessity of prolonged storage of the extracts prior to fractionation, acetal phosphatides, found by Lovern *et al.* (1957) to be present in fresh sperm extracts, were not specifically tested for.

In contrast to the intensive labeling of sperm lipids by carbohydrate precursors, incubation with acetate-1-C^{14} as the only added substrate or in combination with unlabeled glycerol resulted in only slight labeling of the sperm lipids (Table III). Again the diglycerides contained most of the radioactivity, which was recovered in the methyl esters of their fatty acids, whereas the glycerol moiety appeared to be unlabeled. Only slight but measurable radioactivity was found in the phosphatide fractions of spermatozoa incubated with acetate-1-C^{14} as the only radioactive substrate.

TABLE III

LABELING OF LIPID FRACTIONS OF BULL SPERMATOZOA

Washed bull spermatozoa incubated under aerobic conditions with acetate-1-C^{14} (2.5×10^{-3} M) and nonradioactive glycerol (1.25×10^{-3} M). The dialyzed lipid extract (350.4 mg; specific activity 22 cpm/mg) on partitioning between hexane and 90% methanol yielded fraction A (153 mg; specific activity 26 cpm/mg) and fraction B (184 mg; specific activity 22 cpm/mg). Further details as in Table II.

Column	Fraction No.	Solvent	Wt. of Fraction (mg)	Specific Activity (cpm/mg)
A	1	Hexane	17.3	16
	2	Hexane–3% ethyl ether	3.3	5
	3	Hexane–10% ethyl ether	30.2	6
	4	Hexane–25% ethyl ether	14.2	48
	5	Hexane–ethyl ether (1:1 v/v)	14.6	16
	6	Ethyl ether	2.0	13
	7	Methanol–ethyl ether (1:3 v/v)	12.6	14
	8	Methanol–ethyl ether (1:1 v/v)	6.0	18
	9	Methanol–chloroform (1:1 v/v)	12.4	45
	10	Methanol	1.9	17
B	1	Benzene	157.5	13
	2	Benzene–ethyl acetate (9:1 v/v)	4.7	40
	3	Benzene–ethyl acetate (1:1 v/v)	1.3	11
	4	Ethyl acetate	0.9	40
	5	Ethyl acetate–methanol (1:1 v/v)	4.0	17

Mono- and triglycerides and cholesterol esters were not further examined, since little radioactivity appeared in the chromatographic fractions containing those substances after incubation of sperm suspensions with the various radioactive substrates.

C^{14}-Labeled Ketosteroids in Bull Spermatozoa.—Four radioactive Zimmermann-positive zones were separated from the fractions containing diglycerides and cholesterol. Their mobilities on repeated paper chromatography in the systems of Zaffaroni (Burton *et al.*, 1951) and Bush (1952)

[1] We wish to thank Dr. Erich Baer for the gift of samples of dimyristoyl lecithin and dimyristoyl cephalin.

corresponded to the mobilities of certain known steroids which were run as standards at the same time (Table IV). No claim of identification can be made at present, however, because of disparities between the infrared spectra of the unknown and the standard materials. Since the material was isolated from combined extracts of spermatozoa which had been incubated with various C^{14}-labeled substrates, no conclusion is drawn from their relative specific activities other than that the material seemed to have retained its radioactivity throughout the purification procedures. Testosterone was not present in extracts of incubated spermatozoa, although it was found in the seminal plasma which had been separated from the spermatozoa prior to their incubation.

TABLE IV

C$_{19}$-KETOSTEROIDS IN BULL SPERMATOZOA

The corresponding Zimmermann-positive zones recovered from paper chromatograms of extracts of spermatozoa incubated with glycerol-1-C^{14}, glucose-1-C^{14} (randomly labeled), and acetate-1-C^{14} were combined and further purified by paper chromatography (Bush, 1952). The zones are numbered in decreasing order of mobility.

Zone No.	UV Absorption on Paper	Specific Activity (cpm/mg)	Chromatographic Standard of Similar Mobility
1	+	46	Δ^4-cholesten-3-one
2	+	73	Δ^1-androstene-3:17-dione
3	+	90	Δ^4-androstene-3:17-dione
4	−	51	Dehydroepiandrosterone

C$_{21}$-Steroids.—Only one blue tetrazolium–reducing zone was localized on paper chromatograms of the material eluted from column B in fraction 4 (see Tables II and III). This substance absorbed ultraviolet light on paper and had the same R_F value as an authentic sample of cortisol. It was lost accidentally before its radioactivity could be determined.

DISCUSSION

Labeling of the Glycerol and Fatty Acid Portion of Sperm Lipids.—The incorporation of C^{14}-labeled glycerol into lipids by bull spermatozoa which had been incubated with radioactive glycerol or glucose is evidence of the biosynthetic potential of this tissue. The observation that the specific activity of the diglyceride fraction exceeded by far that of the phosphatide and triglyceride fractions is in agreement with the generally accepted view that diglycerides are the precursors of both triglycerides and phosphatides (Kennedy, 1957).

Glucose was as good a source of glyceride-glycerol as glycerol itself. The formation of glycerol-labeled glycerides and phosphatides suggests that spermatozoa may utilize carbohydrate not only for energy for motility, but also for the replenishment of endogenous lipid reserves. Their ability to do so under anaerobic conditions of incubation is of especial interest.

Acetate, however, which is oxidized by bull spermatozoa more readily than glucose (Terner, 1959), was incorporated into the fatty acids of the glycerides at a considerably slower rate than might be expected from the rate of labeling of the glycerol moiety. It is possible that optimal conditions for the synthesis of fatty acids from acetate were not maintained in the present experiments. On the other hand, a similar observation was reported by Cahill *et al.* (1959), who found that in rat adipose tissue the rate of glyceride-glycerol synthesis greatly exceeded that of the fatty acids. Although the diglycerides contained mainly saturated fatty acids at the time of analysis, their relatively low content of unsaturated fatty acids may have been due to partial degradation during the prolonged periods of storage needed to accumulate sufficient sperm material.

Rate of Synthesis of Diglycerides.—From the specific activity of the original substrate (glucose or glycerol) and the amount and specific activity of the diglycerides, the amount of glycerol incorporated into the diglycerides during the standard incubation period of 2 hours was calculated to be of the order of 1 mμmole/10^8 spermatozoa, corresponding to a $Z_{glycerol}$ value of 0.01 ($Z = \mu l/10^8$ spermatozoa/hour: 1 μmole = 22.4 μl). Although the rate of glyceride biosynthesis appears quite low in comparison with the rates of glycolysis and oxidation in spermatozoa (Mann, 1958; Terner, 1959), it may be adequate to maintain the level of endogenous reserves in the presence of glycolyzable or oxidizable substrates.

Endogenous Substrates of Respiration of Bull Spermatozoa.—The suggestion by Lardy and Phillips (1943, 1945) that the respiration of washed spermatozoa (endogenous respiration) may be supported by the oxidation of intracellular phospholipid reserves was discounted by Bomstein and Steberl (1957) but supported by Hartree and Mann (1959, 1960), who, after their discovery of plasmalogen as the principal phosphatide of ram spermatozoa, suggested that the fatty acids of plasmalogens might be the principal source of oxidizable substrate in the absence of carbohydrate.

The present experiments demonstrate the presence in bull spermatozoa of considerable amounts of diglycerides and suggest that the turnover of the diglycerides may be more rapid than that of the phosphatides. From the data given in Table II, it can be calculated that 10^8 spermatozoa contained *ca.* 8 μg (*i.e.*, *ca.* 0.014 μmole) diglycerides, the oxidation of which in the absence of other substrates would account for an oxygen uptake of the order of 15–20 μl/10^8 spermatozoa for 1 hour. The Z_{O_2} values for the endogenous respiration of bull spermatozoa have been reported to range from −5 to −30 (Lardy and Phillips, 1943; Mann, 1958; Melrose and Terner, 1953). Thus in bull spermatozoa diglycerides may be a ready source of fatty acids which may be utilized to support the endogenous respiration.

C^{14}-Labeling of Steroids.—Breuer (1955), using paper chromatographic techniques, found in the "free" fraction of steroids of epididymal bull spermatozoa Δ^4-cholesten-3-one, epiandrosterone

or dehydroepiandrosterone, and etiocholan-3α-ol-17-one. Some doubt remained whether those steroids had been extracted from the interior of the sperm cell or had been held by the fluid adhering externally to the cell walls. In the present experiments the recovery of labeled steroids from the lipid extracts of ejaculated, washed spermatozoa suggests the presence within the cells of enzyme systems capable of catalyzing the synthesis of steroids. Although for technical reasons the products of all incubations with the various C^{14}-labeled substrates were combined prior to final isolation and analysis, it seems obvious that, whether acetate or carbohydrate was the labeled precursor, the building blocks must have been 2-carbon units.

The distinct C^{14}-labeling of ketosteroids in bull spermatozoa contrasts with the absence of measurable radioactivity in the cholesterol isolated. It is conceivable that the specific activity of a small amount of C^{14}-labeled cholesterol merging with the large pool of preformed cholesterol may be reduced to a very low level by isotope dilution. However, it would have to be assumed that the C^{14}-labeled precursors of the ketosteroids bypassed the cholesterol pool.

Testosterone was not detected in extracts of incubated spermatozoa. Its absence from fresh epididymal spermatozoa had been noted by Breuer (1955).

It would be difficult at present to ascribe a function to the steroids produced by mature spermatozoa. Possibly this biosynthetic capacity of the spermatozoa may be a residual property of earlier developmental stages in the life cycle of the germ cell.

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