INFLUENCE OF TEMPERATURE ON THE INCORPORATION OF PALMITIC ACID-U-¹⁴C INTO RAT TESTICULAR LIPIDS

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Summary. Biosynthesis of protein in rat testes is known to have a temperature optimum at 32° C; the normal scrotal temperature. The incorporation of palmitic acid into lipids was examined for a temperature dependence. Testicular tissue was incubated for 2 hr with palmitic acid-U-¹⁴C at 28, 30, 32, 34 and 36° C. Lipids were extracted and lipid classes separated by thin-layer chromatography. Palmitic acid oxidation was measured. Most lipids incorporated palmitic acid with a positive temperature coefficient. The percentage increases in incorporation of fatty acid over the 8° C range were: free fatty acid (58), triglycerides (51), sterol esters (56), diglycerides (19), monoglycerides (marked decline), sterols (no change), phosphatidyl choline (66), lysophosphatidyl choline (36), cephalins (25). The percentage distribution of neutral lipid was free fatty acid (9), triglycerides (4), sterol esters (29), diglycerides (6), monoglycerides (6), sterols (25). The percentage distribution of phospholipid was: phosphatidyl choline (41), lysophosphatidyl choline (3), cephalin (44), sphingomyelin (4). Palmitic acid oxidation increased 13% over the 8° C range. It is concluded that lipid synthesis is not as sensitive to temperature change as protein synthesis. Adverse effects of temperature on lipids are probably a consequence of impaired protein metabolism.

INTRODUCTION

It has been known for some time that normal spermatogenic activity is facilitated by the lower ambient temperature provided by the scrotal position of the testes in most mammals (Moore & Quick, 1924; Moore, 1924). Recently, it has been found that the ability of rat and rabbit testes to incorporate lysine into protein is maximal at their respective scrotal temperatures (Hall, 1965; Davis, Firlit & Hollinger, 1963) and similar findings have been extended to other species (Buyer & Davis, 1966). The production of testosterone from acetate in rabbit testes is subject to the same temperature sensitivity as protein synthesis and in-vivo or in-vitro treatment with interstitial cell stimulating

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hormone does not negate the temperature optimum (Hall, 1965). Elevation of the temperature of the rabbit testis *in vivo* for 24 hr results in a significant decline in oxygen uptake and glucose and lactic acid content (Ewing & Van-Demark, 1963a, b). A histochemical study of short-term temperature effects on several rat testicular enzymes has revealed no marked changes in enzyme content (Turpeinen, Turpeinen & Talanti, 1962) whereas another study employing experimental cryptorchidism has demonstrated a marked decrease in the content of steroid-3B-ol dehydrogenase (Kormano, Harkonen & Kontinen, 1964).

Studies are now in progress in this laboratory to determine the influence of temperature on various metabolic processes in rat testes. The present report contains a study on the effect of temperature on the incorporation of palmitic acid- $U-^{14}C$ into the neutral lipid and phospholipid components of rat testes. Previous studies on rat testicular lipids have been concerned with the content of total lipids, phospholipids, cholesterol and fatty acid patterns (Bieri & Andrews, 1964; Bieri & Prival, 1965; Hall, Nishizawa & Eik-Nes, 1963).

EXPERIMENTAL PROCEDURE

Incorporation of palmitic acid- $U-^{14}C$ into lipids

Holtzman rats, weighing 200 to 300 g, were used throughout and were maintained on Wayne Lab-Blox, water and 14 hr of daylight/day.

Approximately 200 mg of decapsulated testicular tissue was incubated for 2 hr in 0.05 M tris-HCl buffer (pH 7.5), containing 3 g bovine albumin/100 ml of buffer, with palmitic acid- $U-^{14}C$ (1.0 μ C in 0.1 ml propylene glycol; 93 mc/mm). The final volume was 3.1 ml. Twenty-five-ml Erlenmeyer flasks were incubated in an Eberbach metabolic shaker and gassed continuously with 95% oxygen and 5% carbon dioxide. At each temperature three experiments were performed consisting of four flasks for each animal.

Following the incubation period, the tissue from each animal was pooled, homogenized in chloroform-methanol (2:1) and the lipids extracted (Folch, Lees & Sloane-Stanley, 1957). The lipid extract was flash evaporated, washed with methanol and brought to a final volume of 0.3 ml with chloroform-methanol (2:1).

Thin-layer chromatography

Silica gel G plates, 250 μ thick and activated at 110° C for 4 to 8 hr, were used throughout. For the separation of neutral lipids the solvent system used was *n*-pentane-diethylether-acetic acid (90:10:1) and for phospholipids, chloroform-methanol-water (65:35:4). This chromatographic system is essentially that developed by Bowyer, Leat, Howard & Greshaw (1963) except that light petroleum (b.p. 40 to 60° C) was replaced by *n*-pentane and the plates were activated for a longer time and at a higher temperature. As a result of these modifications it is possible to distinguish the monoglyceride component a short distance above the origin since the phospholipids do not streak. Phospholipids were also separated with a two-way system consisting of chloroform-methanol-7 N ammonium hydroxide (60:35:5) followed by chloroform-methanol-

7 N ammonium hydroxide (35:60:5) (Skidmore & Entenman, 1962). For radio-activity determinations, 0.025 ml of the final lipid extract was spotted in a band for both the neutral and phospholipids. For chemical assay 0.025 ml was spotted for the phospholipids and 0.10 ml for the neutral lipids. The bands were visualized with iodine vapour and each band was scraped from the plate after dissipation of the iodine and eluted with chloroform-methanol (2:1). Those bands used for determination of radio-activity were scraped directly into polyethylene vials to which was added 10 ml of scintillation fluid (Bray, 1960); in the case of the phospholipids the scintillator contained 4 g thixotropic gel powder (Packard)/100 ml scintillator.

Chemical assays

Each neutral lipid fraction was assayed for total organic matter according to the method of Johnson (1949) using cholesterol as a standard. The results are expressed as the percentage of total neutral lipids/fraction. The phosphorus content of each phospholipid fraction was determined using a modified assay (Hill, 1966), employing the digestion procedure of Harris & Popat (1954) and the colour developing reaction of Dryer, Tammes & Routh (1957). Briefly, the dried lipid sample was digested at 190° C in a dry bath with 70% perchloric acid followed by addition of 0.04 ml of concentrated nitric acid. Colour was developed by adding 2.0 ml of semidine hydrochloride solution (N-phenyl-*p*-phenylene-diamine monohydrochloride). The results are expressed as the percentage of total lipid phosphorus/fraction.

Conversion of palmitic acid-U-¹⁴C to carbon dioxide

Approximately 400 mg (two pieces) of testicular tissue was incubated in 100 ml respirometers for 2 hr in the tris-HCl-albumin buffer with 2.0 µc palmitic acid-U-¹⁴C. The 5.0 ml centre-well contained 2.0 ml of hydroxide of Hyamine (Packard) and a filter paper collar; each flask was gassed for 30 sec with 95% oxygen and 5% carbon dioxide before incubation. The reaction was stopped by injecting 2.0 ml of concentrated HCl followed by an additional 1 hr of shaking. The carbon dioxide absorbant (0.5 ml) was removed from the centre-well for scintillation counting.

RESULTS

Text-figs. 1 and 2 indicate the effect of temperature on the incorporation of radio-active palmitic acid into the neutral lipid and phospholipid fractions. The uptake of free fatty acid increased 58% over the 8° C temperature range; the accumulation products, triglycerides and sterol (cholesterol) esters increased 51% and 56%, respectively. Diglycerides showed a moderate increase in activity (19%) and the level of incorporation is high relative to the mono- and triglycerides. Overall, monoglycerides showed a marked decline in fatty acid incorporation, but this result is not reliable since this component is easily contaminated with small amounts of phospholipids of high activity; the peak at 32° C has been attributed to this problem. The sterol activity remained essentially constant throughout the experiment. The unknown band, X-1, ran at the solvent front in the neutral lipid system and has not been identified.

Among the phospholipids identified, the band corresponding to phosphatidyl choline showed the greatest increase in fatty acid incorporation from 28 to 36° C (66%) followed by lysophosphatidyl choline (36%) and cephalin (25%). A clear effect of temperature on the sphingomyelin fraction could not be demonstrated. Although a band corresponding to cardiolipin was resolved, the radio-activity recovered was extremely variable due to neutral lipid contamination. The cardiolipin phosphorus content was $3.74 \pm 0.8\%$ and radio-activity $21,379 \pm 2853$ counts/min/g fresh tissue.

TEXT-FIG. 1. Incorporation of palmitic acid- $U-^{14}C$ into neutral lipids after 2 hr of incubation. Each point represents the average for three different animals. FFA, free fatty acid; D, diglyceride; SE, sterol ester; T, triglyceride; S, sterol; M, monoglyceride; X_1 , unknown.

A preliminary determination of the total lipid and phospholipid content of the rat testis gave values in an agreement with the findings of Bieri & Prival (1965); total lipid: 18.8 mg/g fresh tissue, and phospholipid: 13.7 mg/g fresh tissue. The results of the total organic solids assay are given in Text-fig. 3. Since no net quantitative increase in the lipid components could be demonstrated, the values in Text-fig. 3 represent the sum of all determinations regardless of the incubation temperature. Almost 54% of the neutral lipids was represented by sterols and sterol esters, approximately 6% each by mono- and diglycerides whereas the triglyceride fraction accounted for only 4% of the total. If the sterol fraction is composed chiefly of cholesterol, it can be estimated, on the basis of a fatty acid analysis of rat testes (Bieri & Prival 1965), that the

TEXT-FIG. 2. Incorporation of palmitic acid-U-¹⁴C into phospholipids after 2 hr of incubation. Each point represents the average for three different animals. PC, phosphatidyl choline; PE, cephalin; LPC, lysophosphatidyl choline; Sph, sphingomyelin.

TEXT-FIG. 3. Percentage distribution of neutral lipid components (open columns) and radio-activity (shaded columns), after a 2 hr incubation. Since no quantitative change was noted in the distribution of components as a function of temperature, each bar represents the sum of all determinations regardless of the incubation temperature. Standard error of the mean is indicated by the length of the vertical lines; number of animals = 14. For symbols see Text-fig. 1.

TEXT-FIG. 4. Percentage distribution of phospholipid components (open columns) and radio-activity (shaded columns), after a 2 hr incubation. Determinations were grouped as in Text-fig. 3. Number of animals = fifteen. For symbols see Fig. 2. Cph, cephalin.

TEXT-FIG. 5. Conversion of palmitic acid-U- ^{14}C to carbon dioxide after a 2 hr incubation. Each point represents the average of three determinations for each of three different animals. Standard error of the mean is indicated by the length of the vertical lines.

total cholesterol content is around 40% of the neutral lipids and the ratio of free to esterified cholesterol about 1.65.

The distribution of phosphorus in the phospholipids is shown in Text-fig. 4. It can be seen that phosphatidyl choline and cephalin represent 85% of the testicular phospholipids. The results reported here do not agree with a previous report employing paper chromatography. Bieri & Andrews (1964) find almost twice as much phosphorus in phosphatidyl choline as in cephalin and their value for sphingomyelin is four-fold larger. Two-way chromatography has shown that the sphingomyelin fraction contained phosphatidyl serine; no phosphatidyl inositol was found in the phosphatidyl choline fraction.

The final experiment was concerned with the ability of rat testicular tissue to oxidize palmitic acid. It is evident from Text-fig. 5 that palmitic acid is readily converted to carbon dioxide and is associated with a 13% increase in activity over the 8° C temperature range.

DISCUSSION

It is clear from these results that incorporation of palmitic acid into rat testicular lipids increases uniformly from 28° to 36° C rather than responding with a maximal incorporation at 32° C as has been shown for protein and testosterone synthesis (Hall, 1965). It is likely, however, that a temperature optimum may occur as a secondary effect of impaired protein synthesis after prolonged treatment with temperatures above or below the optimum.

The high rate of incorporation of fatty acid into diglyceride, phosphatidyl choline and, to a lesser extent, cephalin fractions suggests that the testis actively metabolizes phospholipids. Hartree & Mann (1961) have presented evidence that washed ram spermatozoa are capable of utilizing plasmalogen as a fatty acid donor to fulfil oxidative energy requirements. Since palmitic acid conversion to carbon dioxide has been demonstrated in this report as well as by others (Fritz, 1964), it is evident that oxidation of fatty acids released from phospholipids could be taking place in germinal epithelium and other tissue elements as well.

Terner & Korsh (1962) have studied the incorporation of labelled glucose and acetate into lipids of isolated bull spermatozoa. They report that most of the substrate in phospholipids appeared in phosphatidyl choline after a 2-hr aerobic incubation and diglycerides incorporated label much more readily than mono- or triglycerides. Other lipid fractions incorporated small amounts of substrate. It is evident from the data given in the present report that the lipids of rat testicular tissue show incorporation characteristics much like those of motile bull spermatozoa. However, it should be pointed out that immobilization of bull spermatozoa with formaldehyde completely suppressed lipid biosynthesis. Since neither the reproductive nor endocrine elements within the testis are motile, it is of interest that lipids are synthesized as actively as in mature spermatozoa. It is more likely that the biosynthesis of phospholipids in the testis is a reflection of membrane turnover in the several testicular cell-types rather than synthesis for utilization as a source of oxidative energy as appears to be the case in spermatozoa isolated from seminal plasma.

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