Elevated Cholesterol and Dolichol Synthesis in Mouse Pachytene Spermatocytes*

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Results are presented here which demonstrate that the rates of $[^{14}C]$acetate incorporation into cholesterol and dolichol increased 4- to 5-fold as mouse spermatocytes matured from the preleptotene to prepuberal pachytene stages. The rate of acetate incorporation into cholesterol then decreased in late pachynema, remained low at all subsequent stages of meiosis, and was very low in mature sperm. In contrast, the rate of acetate incorporation into dolichol remained elevated in late pachytene spermatocytes and round spermatids, then decreased and remained low in mature sperm. The ratio of the rate of $[^{14}C]$acetate incorporation into dolichol to the rate of incorporation into cholesterol increased during late meiotic prophase and remained high in round spermatids; this altered ratio is further evidence of independent regulation of dolichol and cholesterol synthesis in testes. It was shown previously that normal adult mouse testes incorporated acetate into dolichol at a much higher rate (1.8 to 2.4% of the rate of incorporation into cholesterol) than did testes from sterile $W^W/W^+$ mice (0.02%) or X-irradiated mice (0.24%). This high rate of acetate incorporation into dolichol in adult testes is now attributed to differentiating spermatocytes, with particularly high rates being observed during pachynema.

Phosphorylated derivatives of the isoprenoid lipid dolichol serve as carriers of saccharide residues during the assembly of N-glycosidically linked proteins (1-3). The biosynthetic pathway for dolichol branches from the cholesterol synthetic pathway at the level of farnesyl pyrophosphate, after the rate-limiting enzyme for cholesterol synthesis, 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase), is added to the pathway. An increase in the activity of HMG-CoA reductase in cultured mouse fibroblasts, sublines of NCTC 929 strain L (L-cells) and inhibit sterol synthesis, but high concentrations of inhibitor, which suppress cholesterol synthesis by 75%, are required for appreciable inhibition of dolichol synthesis (7). A similar relationship between the two pathways was observed in mouse liver (8). Thus, although the rates of dolichol and cholesterol synthesis in L cells and liver are controlled by one regulatory enzyme, there is some degree of independent regulation. In several different cell cultures and in liver, the rate of incorporation of $[^{14}C]$acetate into dolichol did not exceed 0.11% of the rate of incorporation into cholesterol (7, 9).

Regulation of dolichol synthesis in two differentiating tissues, developing mouse brain and spermatogenic testes, appears to differ from that in L-cell cultures and liver. At 7 days after birth, cholesterol synthesis was declining in mouse brain yet a peak of dolichol synthesis was observed (10). James and Kandutsch (9) demonstrated that adult mouse testes synthesized dolichol at a rate which was 1.8 to 2.4% of the cholesterol synthetic rate; this ratio of dolichol to cholesterol synthesis was 20- to 72-fold greater than that observed in mouse liver and L-cell cultures. Testes from sterile $W^W/W^+$ and X-irradiated mice had rates of 0.02% and 0.24%, although the rates of cholesterol synthesis were not lower than 50% of the rate in normal testes. Thus, the high rate of dolichol synthesis in mouse testes appeared to be due to the presence of differentiating spermatogenic cells. Acetate incorporation into dolichol has now been measured in purified populations of germ cells from immature and mature mice. The data indicate that the high rate of dolichol synthesis in mouse testes can be attributed to pachytene spermatocytes and to round spermatids.

EXPERIMENTAL PROCEDURES

Materials—The following compounds were obtained from New England Nuclear Corp. R.S.3-hydroxy-3-methyl[3$^-^{14}C$]glutaryl-CoA (56 Ci/mmol), $[^{1-14}C]$acetic acid, sodium salt (57 Ci/mmol), $[^{1,2-3H}]$cholesterol (40 to 60 Ci/mmol), $[^{1-3H}]$dolichol (12.5 Ci/mmol), $[^{3H}]$mevalonolactone (2 to 10 Ci/mmol). Burdick and Jackson glass-distilled solvents were used for high pressure liquid chromatography. The following materials were obtained from the companies indicated: collagenase, CLS grade, Worthington Corp.; trypsin, bovine pancreas, type III, Sigma Chemical Co.; Nitex nylon mesh, 40 to 80 mesh, Tet Kressilk.

Mice—Adult CD-1 mice aged 60 to 120 days were obtained from Charles River Breeding Laboratories, Wilmington, MA. Adult Tac; (SW)fBR mice of the same age were obtained from Taconic Farms, Inc. (Germantown, NY) and used for some experiments. No difference was detected in the amount of dolichol synthesized from $[^{14}C]$acetate by testes from either strain. All experiments involving mouse spermatogenic cell populations earlier than mid-to-late pachynema were conducted using CD-1 male pups of the appropriate age (11).

Incubation of Testes—Animals were killed by cervical dislocation. Testes were immediately excised and decapsulated with care not to press the organ since pressure increases the number of multinucleated germ cells obtained from cell separation experiments (12). Decapsulated adult testes were teased apart slightly and incubated for 4 h at 33 °C in Krebs-Ringer bicarbonate buffer or phosphate-buffered saline. Krebs-Ringer buffer consisted of 120.1 mM NaCl, 4.8 mM KCl, 25.2 mM NaHCO$_3$, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 7H$_2$O, 1.3 mM CaCl$_2$, 100 μg/ml of streptomycin sulfate, and 60 μg/ml of penicillin G.
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(K⁺ salt), pH 7.5. The enriched Krebs-Ringer buffer containing added glucose and amino acids usually used for mouse spermatogenic cells (13) was not used here because it significantly lowered incorporation of [14C]acetate, presumably by decreasing the specific activity of the intracellular acetate pool. Phosphate-buffered saline consisted of 8.0 g of NaCl, 1.15 g of Na₂HPO₄, 0.2 g of KH₂PO₄, 0.2 g of KCl/liter of H₂O pH 7.4. Rates of incorporation of labeled acetate into dolichol or cholesterol were similar in the two media.

Testes from 10 adult mice were incubated in 10 ml of Krebs-Ringer buffer medium containing 800 μCi of [14C]acetate. For assay of prepuberal spermatogenic cells, 160 unteased testes were incubated in 5 ml of buffer containing 400 μCi of [14C]acetate. Before incubation, the flasks were flushed with air-CO₂ (95:5). After incubation, all testes were washed three times by gravity sedimentation in enriched Krebs-Ringer buffer (Krebs-Ringer buffer containing 1.1 mM glucose, 10 ml/liter of essential amino acids, and 10 ml/liter of nonessential amino acids). Enriched Krebs-Ringer buffer was used then for all subsequent stages of cell separation.

Separation of Spermatogenic Cells—The overall experimental plan is diagrammed in Fig. 1. Purified populations of mouse spermatogenic cells were obtained using established procedures (12, 13). Testes washed free of exogenous [14C]acetate were first incubated in 0.3 mg/ml of collagenase for 12 to 15 min at 33 °C in enriched Krebs-Ringer buffer maintained at pH 7.5 on an oscillating shaker at approximately 120 rpm. This treatment effectively releases long stretches of intact seminiferous tubules from the somatic testicular interstitium. The interstitium consists predominantly of blood cells, macrophages, and cells of the interstitial cell type, cells loosely associated with free, viable germ cells which spill from the broken ends of seminiferous tubules, were obtained from the collagenase-containing supernatant layer by centrifugation at 200 × g for 5 min using a TJ-6R refrigerated centrifuge. Leydig cell enrichment in these preparations averaged 17%. Cells released into collagenase supernatant layers of adult testes also included low numbers of testicular spermatocytes. Testes from prepuberal animals contain no testicular spermatozoa. Isolated seminiferous tubules, essentially consisting only of Sertoli cells and developing germ cells were washed three times in enriched Krebs-Ringer buffer and then incubated in 0.5 mg/ml of trypsin using the same conditions as for collagenase. Trypsin treatment is required (a) to obtain single cell suspensions of developing germ cells which normally are interconnected by wide intercellular cytoplasmic bridges and (b) to remove Sertoli cells. The efficiency of trypsinization and the problems associated with mechanical dissociation of the mammalian seminiferous epithelium have been discussed in detail by previous investigators (12-14). Sertoli cells of the adult seminiferous epithelium are normally joined by extensive tight junctions and are not separated by trypsinization under the conditions used here. Accordingly, Sertoli cell clumps may be removed by filtration on Nitex nylon mesh. In addition, early germ cells, predominantly spermatogonia and spermatocytes under the Sertoli cell tight junctions, were removed at this stage. Sertoli cell junctions of the 17- to 18-day-old mouse testis have not yet fully formed (15), but are developed enough to allow the Nitex filtration step. Germ cell suspensions were then pelleted, washed three times in enriched Krebs-Ringer buffer containing 0.5% w/v bovine serum albumin and separated by unit gravity sedimentation according to established procedures (11-13). In the present study averages values for cell purities and viabilities as determined by trypan blue dye exclusion, were as follows: preleptotene spermatocytes (91% pure, 97% viable), leptotene/zygotene spermatocytes (95%, 98%), preleptotene spermatocytes (94%, 95%), adult spermatocytes (91%, 98%), round spermatids (maturity stages 1 to 8) (93%, 97%), and residual bodies (95%, 98%). Morphological criteria used to establish cell identities have been described previously (11, 12). Protein content of the cell types isolated was determined by the method of Lowry et al. (16) using bovine serum albumin as the standard. Purified germ cell populations were pelleted and washed, and 1 ml of 30% ethanolic KOH containing 80 mg of pyrogallol and 5 mg of butylated hydroxytoluene as anti-oxidants was added. Samples were stored at −20 °C before saponification. The samples were saponified at 80 °C for 2 h and extracted with ether as previously described (7). Hydrolysis of dolichyl phosphate and dolichylphosphoryl saccharides to free dolichol may be incomplete under these saponification conditions (17).

Isolated mature spermatocytes were obtained from the vas deferens and washed at least three times in enriched Krebs-Ringer buffer or phosphate-buffered saline before incubation with [14C]acetate. The incubation mixture consisted of 4 to 6 × 10⁶ sperm in 1 ml of phosphate-buffered saline containing 250 μCi of [14C]acetate. After 4 h at 33 °C, the sperm were washed and saponified as already described.

High Pressure Liquid Chromatography—The high pressure liquid chromatography system used in this study employed a 5-μm C18 reverse phase column (Waters Associates) which was previously described (7). The solvent system used was concave gradient No. 10 (Waters Associates, model 600 Solvent Programmer) from 100% methanol to 100% methylene chloride over 48 min. The flow rate was 2 ml/min. The nonnanoplinifiable lipids, containing [1H]cholesterol and pig liver [3H]cholesterol standards, were fractionated, and the cholesterol- and dolichol-containing fractions were collected.

Measurement of [14C] Incorporation into Cholesterol and Dolichol—The dolichol fraction collected by high pressure liquid chromatography was acetylated and chromatographed on thin layer chromatography silica gel plates with toluene as solvent; each plate was divided into 1-cm bands, and [14C] and [3H] in acetylated dolichol were determined. Overlap of [14C] into the [14C] counting channel was less than 0.05%. Radiolabeled cholesterol was determined as digitonin-precipitable sterol (18).

3-Hydroxy-3-methylglutaryl-CoA Reductase—The activity of HMG-CoA reductase was assayed using purified preparations of germ cells which were stored at −90 °C prior to assay. The cell pellets were thawed in a solution containing 50 mM potassium phosphate buffer, pH 7.4, 5 mM dithiothreitol, 2.5 mM NADP, 20 mM glucose 6-phosphate, 0.2 unit of glucose 6-phosphate dehydrogenase, 80 μM NADPH, 3 mM MgCl₂, 40 μM R-4-Hydroxymethyl[14C]-glutaryl-CoA (20 pmol), and enzyme preparation in a final volume of 50 μl. After incubation for 30 min at 37 °C, 5 μl of [14C]-mevalonolactone (15 × 10⁶ dpm) and 5 μl of 12 N HCl were added. After 30 min at 37 °C, the entire sample was placed onto the sample loading area of one channel of a Whatman LK6D precoated silica gel plate (20 × 20 cm, 19 channels). After loading, the plate was placed face down on a bed of silica gel desiccant in a dry chamber for 1 h. The plate was then developed (ethyl acetate:acetonitrile, 2:1), air-dried, and sprayed with Rhodamine B. The mevalonolactone band was visualized under long wave UV light, scraped into a scintillation...
vial, and 0.25 ml of ethanol and 5 ml of toluene-based scintillation fluid were added. \(^4\)H and \(^{14}\)C in the mevalonolactone band were determined. Protein was assayed using the method of Lees and Paxman (18).

**RESULTS**

Normal mouse testes containing differentiating spermatogenic cells incorporate acetate into dolichol at a rate which is 10- to 50-fold higher than that of testes from immature mice, X-irradiated mice, and sterile W/W\(^a\) mice (9). The results presented here demonstrate that the high rate of dolichol synthesis in normal testes can be attributed to pachytene spermatocytes and to round spermatids. The rates of \(^{14}\)C- acetate incorporation into cholesterol and dolichol in four separate experiments, using cell preparations from two groups of immature mice and two groups of adult mice are shown in Fig. 2. Prepuberal pachytene cells incorporated more acetate into cholesterol than did preleptotene and leptotene/zygotene cells, when the data are expressed as disintegrations per min per 4 h per 10\(^6\) cells; the rates of incorporation then decreased as the cells matured from pachytene spermatocytes to spermatids. The protein content of the spermatogenic cells (Fig. 3) increased between the preleptotene and the adult pachytene stages then decreased, reflecting the two meiotic divisions which give rise to round spermatids. Expression of the data for acetate incorporation as disintegrations per min per 4 h per mg of protein is shown in Table I. The rate of incorporation into dolichol was higher in prepuberal pachytene spermatocytes than at earlier stages, and the rate was low in mature sperm. Incorporation of acetate into cholesterol is also shown as disintegrations per min per 4 h per mg of protein in Table I. The difference in the actual rates of acetate incorporation into cholesterol in the preleptotene, leptotene/zygotene, and prepuberal pachytene cells in experiments 1 and 2 may be caused by slight variations in the age of the mice, in the stage of maturation of the cells of each type, and in the experimental conditions.

In each experiment, the ratio of disintegrations per min in dolichol to disintegrations per min in cholesterol increased as the cells matured from prepuberal spermatocytes to round spermatids.

**Table I**

| Experiment 1 | Experiment 2 |
|--------------|--------------|
| **[\(^{14}\)C]Acetate incorporation** | **[\(^{14}\)C]Acetate incorporation** |
| **Cholesterol** | **Dolichol** | **dpm in dolichol/ dpm in cholestrol x 100** | **Cholesterol** | **Dolichol** | **dpm in dolichol/ dpm in cholesterol x 100** |
| Preleptotene | 7,000 | 110 | 1.6 | 42,000 | 280 | 0.7 |
| Leptotene + zygotene | 9,000 | 110 | 1.2 | 36,000 | 170 | 0.5 |
| Prepuberal pachytene | 8,000 | 270 | 3.4 | 51,000 | 330 | 0.7 |
| Adult pachytene | 5,300 | 160 | 3.0 | 7,100 | 160 | 2.3 |
| Round spermatids | 9,800 | 400 | 4.1 | 9,500 | 220 | 2.3 |
| Sperm | 1,000 | 30 | 3.0 | 6,300 | 140 | 2.2 |
| Residual bodies | 6,000 | 100 | 1.7 | | | |
During the early stages of spermatocyte maturation, HMG-CoA reductase activity increased, whether expressed as picomoles per min per 10^6 cells or picomoles per min per mg of protein (Fig. 4). Reductase activity was low in residual bodies when expressed as picomoles per min per 10^6 cells, but appeared higher when calculated as picomoles per min per mg of protein. However, residual bodies cannot be regarded as complete cells, and the protein content is lower than that of maturing spermatocytes. A lack of metabolic activity in residual bodies has been reported.

**Discussion**

The results presented here demonstrate that prepuberal pachytene spermatocytes incorporate acetate into dolichol at a rate which is 5 times higher than that of purified preleptotene spermatocytes, and 2 to 5 times higher than that of leptotene and zygote spermatocytes, and that a high rate of acetate incorporation into dolichol is maintained in adult pachytene spermatocytes and round spermatids. The rate of acetate incorporation into cholesterol was also high in prepuberal pachytene spermatocytes, and returned to a very low value in mature sperm. There was an increase in the ratio of acetate incorporation into dolichol to incorporation into cholesterol in the pachytene and round spermatid stages. Such elevated ratios were not observed in liver (9) or in proliferating cell cultures (7). These data provide further evidence of independent regulation of dolichol and cholesterol synthesis in mouse testes, and provide a third example of such independent regulation in differentiating mammalian tissues. In developing mouse brain, at approximately 7 days after birth, a peak of dolichol synthesis occurred while the rate of cholesterol synthesis was decreasing (10). During phenylhydrazine-induced erythropoiesis, the ratio of dolichol to cholesterol synthesis in the mouse spleen increased 20- to 30-fold (24).

Cell proliferation, as measured by DNA synthesis, is associated with (20) and, in at least some cell types, dependent on (21), an elevated rate of cholesterol synthesis. One of the functions of the cholesterol is to serve as a component of new cell membrane. Although the enhanced rate of acetate incorporation into cholesterol observed here during the leptotene, zygote, and prepuberal pachytene stages is not associated with DNA replication, it correlates with differentiation events occurring in these spermatocytes. Germ cell diameter (11) and surface area (22) increase between the preleptotene and pachytene stages, so there is a need for synthesis of new plasma membrane. In contrast, late pachytene spermatocytes do not increase much further in size, but differentiate into diplotene spermatocytes which quickly undergo two meiotic divisions to form round spermatids.

The dolichol-dependent pathway for protein glycosylation is essential for the production of specific membrane glycoproteins. Gahmberg et al. (25) showed that tunicamycin inhibited the N-glycosylation of glycoprotein A in the human cell line K562, causing a lower molecular weight variant of the glycoprotein to appear on the cell membrane. Chapman et al. (26) demonstrated that class E mutant Thy-1-negative mouse lymphoma cells are unable to synthesize dolichylphosphorylmannose. These cells produced a structurally defective glycoprotein which did not reach the cell surface; the defective synthesis could be corrected with exogenous dolichylphosphorylmannose as measured in vitro assays. These studies are two examples of the increasing evidence that the assembly of physiologically important membrane glycoproteins is dependent on the dolichol pathway.

The identity of proteins which are glycosylated by dolichol intermediates during mouse spermatogenesis is not yet known, but the high rate of acetate incorporation into free dolichol and dolichyl acyl esters exhibited by specific classes of spermatogenic cells suggests that the dolichol-dependent pathway may be of significance in the regulation of spermatozoan differentiation. This hypothesis is supported by the work of Wastrom and Hamilton (27) who reported that the distribution of dolichol in the reproductive tract of male rats corresponds to spermatocyte maturation, with the highest concentration of dolichol and the highest rate of [4C]mevalonate incorporation into dolichol being in the corpus epididymis. Isolated mouse spermatogenic cells have relatively high amounts of plasma membrane glycoproteins, as assayed in lectin-binding studies (22) and as determined biochemically using affinity chromatographic isolation techniques. In addition, the plasma membranes of mature mammalian spermatooza contain glycoproteins which (a) are localized and restricted to distinct regions of the cell surface (28, 29, reviewed by 30) and which (b) change both qualitatively and quantitatively during sperm maturation and transit in the male reproductive tract (31-33). Biochemical procedures for the isolation of these surface components have been developed in some instances (34, 35) but few experiments relating to the glycosylation of spermatogenic cell proteins have been reported to date. Letts et al. (36) have measured the activities of three glycosyltransferases in purified mouse spermatogenic cells.

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2 M. J. James, unpublished data.

3 C. F. Millette, unpublished observations.
finding that glycosyltransferase levels in spermatocytes and early spermatids were high. These results, as well as those presented here, suggest that changes in various glycoproteins may be important during spermatogenesis.

Serological investigations of spermatogenic cell surface constituents demonstrate, moreover, that newly expressed differentiation antigens appear on developing germ cell plasma membranes. Specifically, new cell surface components, presumably proteins, have been detected on late pachytene spermatocytes and round spermatids of the mouse (14), rabbit (37), rat (38), and guinea pig (39). Recent biochemical comparisons between purified plasma membranes of pachytene spermatocytes and round spermatids have also identified stage-specific differentiation markers during mouse spermatogenesis (40). Few if any, of these surface molecules have been purified and examined in detail and their extent of glycosylation is not known. O’Rand and Porter (35) have isolated a dialyglycoprotein from the membranes of rabbit spermatzoa, but its identity with the other serologically described surface proteins remains to be determined. It is possible that the increased rate of acetate incorporation into dolichol seen in pachytene spermatocytes and round spermatids is associated with the glycosylation and membrane insertion of particular spermatogenic cell differentiation marker proteins. The availability of purified spermatogenic cell populations and isolated plasma membrane fractions from these cells should allow direct examination of this hypothesis.

Finally, it is also conceivable that dolichol in late spermatogenic cells is involved in the glycosylation of acrosomal enzymes. The acrosome is a specialized collection of hydrolytic enzymes that may be glycosylated via the dolichol-dependent pathway (47, 48). Increased acetate incorporation into dolichol by late pachytene spermatocytes and by round spermatids may, therefore, be involved in acrosomal enzyme biosynthesis and packaging. The temporal relationships between acrosomal enzyme synthesis and the appearance of a morphologically identifiable acrosome per se have not been determined.

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