

THE EFFECTS OF TEMPERATURE AND GLUCOSE ON
PROTEIN BIOSYNTHESIS BY IMMATURE
(ROUND) SPERMATIDS FROM RAT TESTES

MASAHISA NAKAMURA, LYNN J. ROMRELL, and PETER F. HALL

From the Department of Physiology, California College of Medicine, University of California, Irvine, Irvine, California 92717 and the Department of Anatomy, College of Medicine, University of Florida, Gainesville, Florida

ABSTRACT

A method is described for the preparation of highly purified fractions (>80% pure) of immature spermatids (round, steps 1-8) from rat testes by centrifugal elutriation in sufficient yields for biochemical studies when four rat testes are used. Electron microscopy established the identity of the cells and demonstrated that the cell membrane is intact. Some cells develop nuclear and cytoplasmic vacuoles during the 2 h required for preparation. Immature spermatids prepared by this method use glucose with an increase in oxygen consumption, lactate production, and protein synthesis over control levels (no glucose). The testicular cell suspension from which spermatids are separated, like whole testis and spermatids themselves, show higher incorporation of amino acids into TCA-precipitable material at 34°C than at 38°C and in the presence of glucose. A subcellular system prepared from immature spermatids with excess ATP shows greater incorporation of amino acids into TCA-precipitable material at 34°C than at 38°C. This difference does not result from increased breakdown of protein. It is concluded that body temperature (38°C) inhibits some aspect(s) of protein synthesis in addition to previously reported effects on amino acid transport and production of ATP (Means and Hall, 1969. *Endocrinology*. **84**:285-297.).

KEY WORDS spermatids · testes · centrifugal elutriation · electron microscopy · protein synthesis · temperature

It has been known for many years that body temperature inhibits spermatogenesis in mammalian testis (6). Davis et al. reported that rat testis shows maximal protein biosynthesis at 32°C in contrast to other organs in which this activity is maximal at or near body temperature (3). Glucose protects the testis from the inhibitory effect of

body temperature on protein biosynthesis (2). Subsequent studies, using indirect approaches, suggested that spermatids are largely but not entirely responsible for the sensitivity of testicular protein biosynthesis to temperature and for the response to glucose (4, 12). A direct approach to the questions of which cell type is affected by temperature and by what mechanism has been provided by modern methods of cell separation which have permitted the preparation of highly purified fractions of spermatids from rat testis

(5, 11). The differentiation of spermatids causes these cells to pass through two grossly different morphological stages which can be readily separated by centrifugal elutriation, namely immature or round forms (steps 1-8 in the rat) and mature or elongated forms (beyond step 8) (11). The studies reported here were designed to characterize the immature spermatid fractions prepared in this manner and to investigate the effect of temperature upon protein biosynthesis in spermatids from rat testes. The characterization of immature spermatids is based upon biochemical and electron microscopic evidence.

MATERIALS AND METHODS

Animals

Rats of the Wistar strain aged 50-60 days were used in these studies. The animals were fed ad lib. before the experiments. Rats were decapitated and the testes removed and placed on ice.

Preparation of Spermatids

The tunica was removed from each testis which was lightly dissected by separating tubules and incubated in a phosphate-buffered saline medium prepared as described by Grabske et al. (5). To this medium, collagenase (0.05%, wt/vol) was added. Incubation was performed at 28°C for 40 min in a metabolic shaker (75 oscillations/min); 40 ml of buffer was used with four testes from two rats (~6 g of tissue). The tissue was then filtered through nylon mesh (61 μ m gauge) and the cells which passed through the mesh were subjected to centrifugal elutriation as described by Grabske et al. (5).

Cells harvested in various fractions from centrifugal elutriation were suspended in 200 ml of buffered saline (as above) and centrifuged at 200 g for 7 min at 4°C in a Sorvall GLC-2 centrifuge (DuPont Instruments, Sorvall Operations, Newton, Conn.). The pellet was resuspended in 20 ml of buffer and transferred to incubation flasks by means of a pipette. The entire preparation from removal of testis to the beginning of incubation required 2 h.

The following studies are based upon immature spermatids (steps 1-8 of spermiogenesis), and these cells will be referred to by that name. Immature spermatids were collected from centrifugation in our fraction 3 (s_{\max} 6.2-7.2). Our fractions from centrifugal elutriation can be compared with those of Grabske et al. on the basis of values for s_{\max} but not by fraction number because their studies used somewhat different conditions and were performed on mouse testis (5).

Preparation for Microscopy

Cell samples were fixed and prepared for light and electron microscopy. Cell suspensions were fixed in 1%

glutaraldehyde in phosphate-buffered saline, pH 7.4, for 1 h, washed in 0.1 M cacodylate buffer, pH 7.4, post-fixed in 1% OsO₄ in the same buffer, dehydrated with ethanol, and embedded in Epon-Araldite. 1- μ m sections were stained with toluidine blue and examined by light microscopy. Thin sections were stained with uranyl acetate and lead citrate and examined with a Siemens Elmiskop 1A.

Number, Identity, and Condition of Cells

Cells were counted in a hemacytometer and protein was determined separately on aliquots from each flask by the method of Lowry et al. (10). It was discovered that relative values for incorporation of amino acids into protein were not significantly different when expressed according to cell number or to protein/flask; in the accompanying studies, values are expressed per milligram of protein. Each flask contained $\sim 8 \times 10^5$ cells (100 μ g of protein). Samples of each preparation of cells were examined by phase-contrast microscopy; other samples for cell counts were air-dried and stained by the periodic acid-Schiff reagent, counterstained with hematoxylin. The percentage of cells which excluded trypan blue was determined with each preparation. Each gradient was loaded with 1.2×10^8 cells (2×10^7 immature spermatids) and the final yield of immature spermatids was 9×10^6 cells (45%).

Protein Biosynthesis

Three methods were used in the following studies to measure the incorporation of amino acids into protein: (a) Precipitation with TCA after incubating cells with a radioactive amino acid. The precipitate was isolated by centrifugation. The details of the method together with evidence that the radioactivity measured in the treated precipitate represents protein synthesis have been given in detail (14). In view of this published evidence, it is convenient to refer to the incorporation of amino acids into TCA precipitate in this system as protein synthesis. For whole cell studies, each flask contained [³H]phenylalanine (2.5 μ Ci; 0.3 μ M). (b) When small numbers of cells were examined, we used Millipore filters (Millipore Corp., Boston, Mass.), instead of centrifugation, to collect and wash the precipitate. The method is in other respects the same as that described under (a). (c) For some studies, a subcellular system was used. Spermatids were subjected to lysis by suspending the final pellet in 0.8 ml of cold distilled water. The cells were then kept on ice for 20 min. The lysate was centrifuged at 24,000 g for 20 min, and the supernate was used in these studies. The lysate (1.3 mg of protein per flask) was incubated with a buffered medium consisting of the following components in a total volume of 0.2 ml, at the final concentrations shown in parentheses: KCl (75 mM); MgCl₂ (2 mM); ATP (0.5 mM); GTP (0.24 mM); creatine kinase (20 U total per flask); creatine phosphate (15 mM); [³H]phenylalanine (5 μ Ci;

0.6 μ M); and an amino acid mixture containing 19 other unlabeled amino acids (0.1 mM each). Incubation was performed at the temperatures shown for 60 min unless otherwise indicated. The reaction was stopped by addition of an equal volume of aqueous TCA (10% wt/vol) containing 0.5 mM phenylalanine. The mixture was heated at 90°C for 15 min, filtered through Millipore filters, and washed with 5% TCA three times. The precipitate was dried and subjected to liquid scintillation spectrometry (7).

Chemicals

The preliminary purification of the [3 H]phenylalanine used in these studies has been given elsewhere (12). The amino acids, L-[ring-2,6- 3 H(N)]phenylalanine (lot number 917-185; sp act 40 Ci/mmol), L-[U- 14 C]tyrosine (lot number 881-181; sp act 435 mCi/mmol), and L-[U- 14 C]lysine (lot number 881-165; sp act 306 mCi/mmol) were obtained from New England Nuclear (Boston, Mass.). Collagenase (type IV) was purchased from Worthington Biochemical Corp. (Freehold, N. J.).

Miscellaneous

Methods for calibrating thermometers and incubating at different temperatures have been given elsewhere (7). Oxygen consumption was measured by incubating spermatids in phosphate-buffered saline (pH 7.4) for 20 min in a volume of 1.5 ml, using a Clark electrode (18). Lactate was measured on samples of medium after incubation of spermatids for 1 h in phosphate-buffered saline (8). The method is capable of detecting 1 μ g of lactate, and duplicate determinations show a standard error of ± 0.065 μ g.

RESULTS

Characterization of Immature Spermatids

Immature spermatids constituted 17% of the cell suspension obtained after treatment with collagenase (Fig. 1a). >98% of the immature spermatids prepared by this method excluded trypan blue. The separated fractions of spermatids recovered after centrifugal elutriation (Figs. 1b and 2) were >80% pure ($82 \pm 4\%$; mean and range for 10 determinations). For these determinations, 500 cells were counted.

MICROSCOPY: Cells fixed immediately after dissociation, i.e., before centrifugation (Fig. 3a) demonstrated morphological features typical of cells observed in intact testis, except that the intercellular bridges which join clones of differentiating germ cells were disrupted. There was also loss of the association between the germ cells and neighboring Sertoli cells. The electron microscopic appearance of the isolated spermatids is consistent

with that observed with other preparations of separated testicular cells (1, 17). The immature spermatids that were recovered as highly enriched cell fractions by centrifugal elutriation demonstrated several subtle changes in their morphology. Nuclear and cytoplasmic vacuolization were observed in most cells immediately after separation (Figs. 2 and 3b). Commonly, there was a general increase in the size of the cells and a swelling of the nuclear envelope, the Golgi apparatus, and mitochondria (suggestive of osmotic shock). Vacuolization of the acrosome was observed in <5% of the spermatids. Even though the mitochondria were somewhat increased in size, they were, in general, of a morphological configuration typical of germ cells at this stage of differentiation. The plasma membrane was intact in almost all of the cells observed. Cells held at 4°C for 2 h after dissociation demonstrated similar morphological changes. By contrast, as mentioned above, such changes were not seen in cells examined immediately after dissociation.

BIOCHEMICAL STUDIES: It can be seen from Table I that immature spermatids consume oxygen at a low but measurable rate which is more than doubled by addition of glucose. The spermatids do not produce detectable amounts of lactate but do so when glucose is added to the incubation medium. Fig. 4 shows that immature spermatids incorporate [3 H]phenylalanine into protein. Incorporation is linear for at least 60 min and is accelerated by addition of glucose to the incubation medium.

The Influence of Temperature on Protein

Biosynthesis by Testicular Cells

WHOLE CELL SUSPENSION: When testicular cells are separated by incubation with collagenase and washed to remove the enzyme, the cell suspension incorporates [3 H]phenylalanine into protein. Incorporation is greater at 30°C than at higher temperatures and is accelerated by addition of glucose (Fig. 5); values for cells incubated at 30°C without glucose were significantly greater at the temperatures tested ($P < 0.001$, $n = 5$). In the presence and in the absence of glucose, temperatures above 34°C cause decrease in incorporation of [3 H]phenylalanine. The cell suspension contains all the various cell types normally present in the testis (11), and incorporation of amino acid into protein resembles that in whole testis with respect to temperature and response to glucose (Fig. 5 and reference 2).

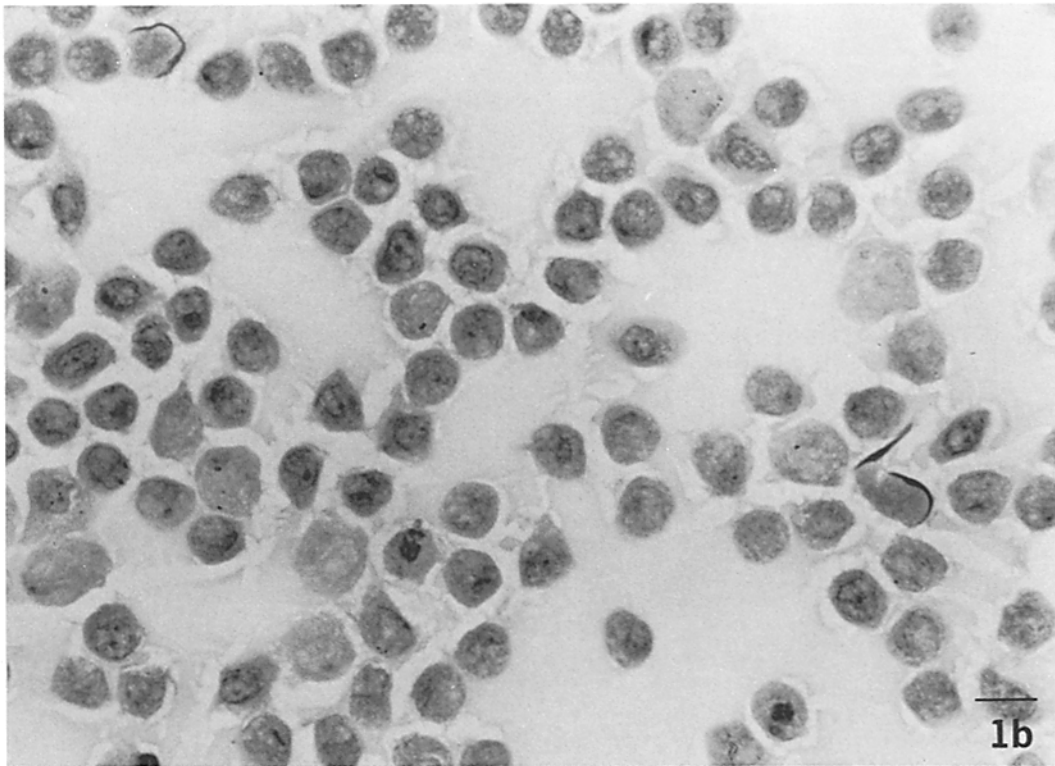
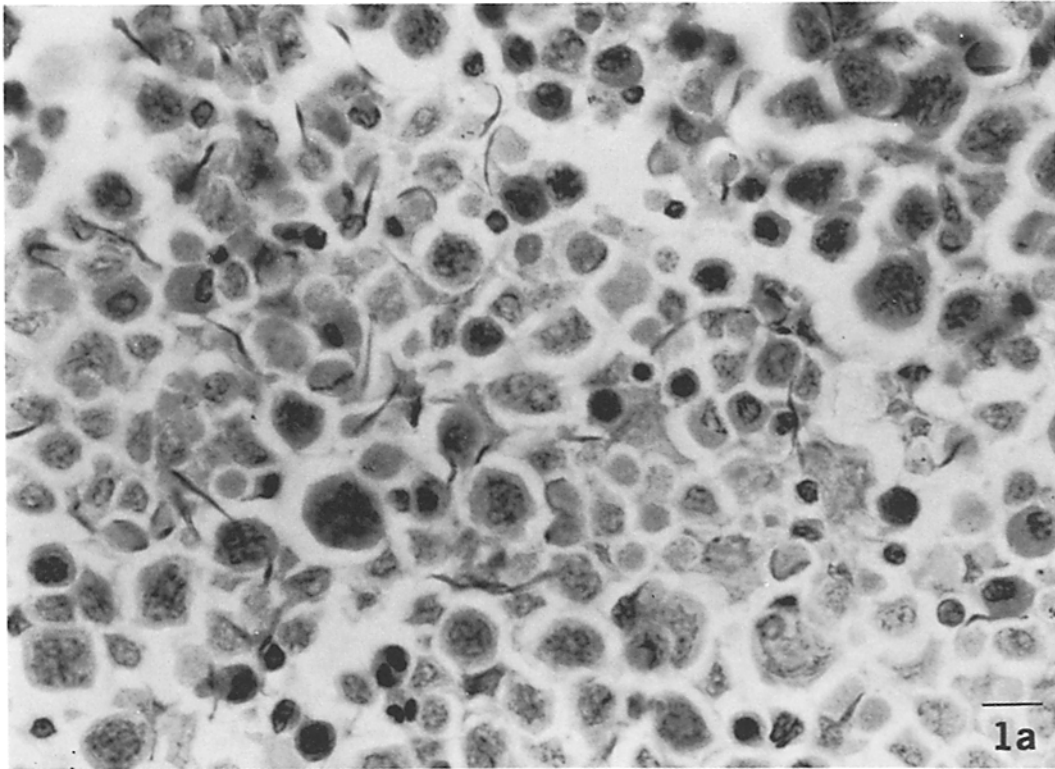


FIGURE 1 Photomicrographs of isolated rat testicular cell smears. Preparations were stained by periodic acid-Schiff reagent, counterstained with hematoxylin. (a) Cell suspension before separation by centrifugal elutriation. (b) Immature spermatid fraction recovered after centrifugal elutriation. The fraction contains >80% immature spermatids (steps 1-8 of spermiogenesis). Bars, 10 μm ; $\times 800$.

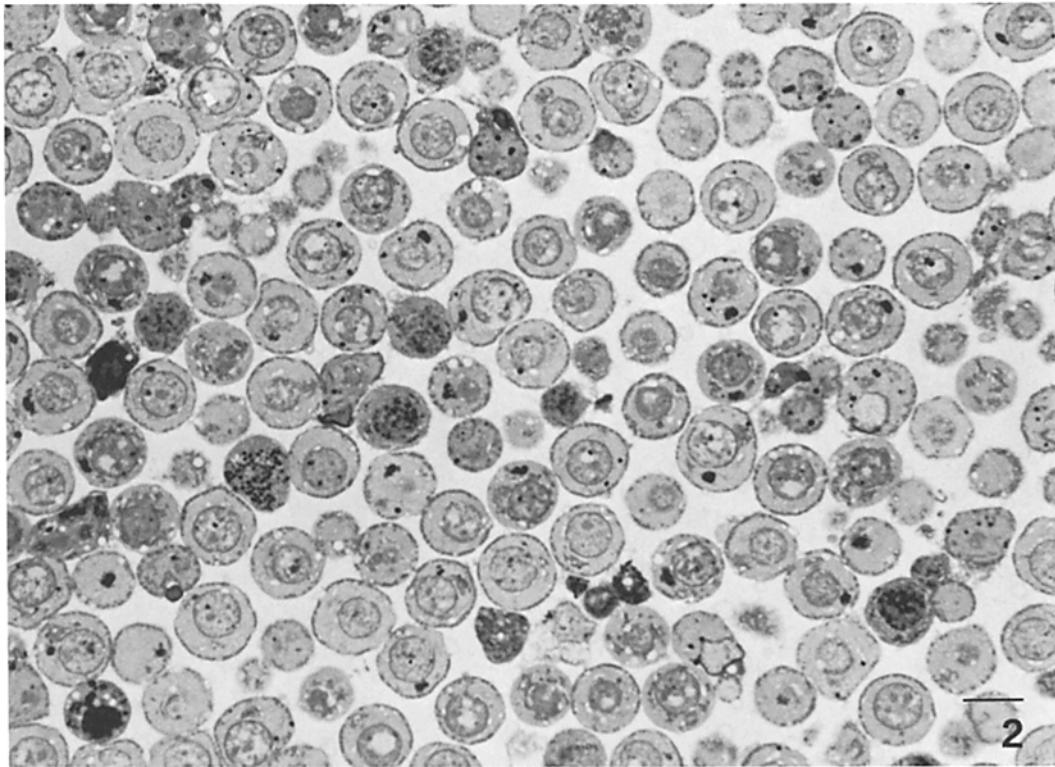


FIGURE 2 Photomicrograph of immature spermatid fraction. 1- μm section stained with toluidine blue. >80% of the cells in this fraction are immature spermatids in various stages of the Golgi phase of acrosome formation. Cytoplasmic and nuclear vacuoles can be seen in many of the isolated cells. Bar, 10 μm ; $\times 800$.

IMMATURE SPERMATIDS: Immature spermatids show decrease in protein biosynthesis at temperatures above 34°C (Fig. 6). Glucose stimulates this process, although the stimulating influence of glucose is less at higher temperatures than at 30° and 34°C. Similar observations were made with incorporation of [^{14}C]lysine and [^{14}C]tyrosine (data not shown).

LYSATE FROM IMMATURE SPERMATIDS: Fig. 7 shows that incorporation of [^3H]phenylalanine into protein by lysate of immature spermatids was greater at 34° than at 38°C. When lysate was incubated for 60 min with all the necessary additions except ATP, very little incorporation of [^3H]phenylalanine was observed (50 cpm/mg of protein). Again, incorporation was dependent upon addition of the 19 unlabeled amino acids and was not increased by addition of poly (U)—presumably because the ribosomes are saturated with endogenous messenger. In preliminary studies, the lysate system was optimized for a number

of variables including pH, divalent metal ions, ionic strength, and concentrations of amino acids. A wide range of concentrations of ATP was examined to exclude effects of ADP on protein synthesis. 10 mM glucose was added without effect. Zero-time controls (addition of TCA) gave values of 50 cpm/mg of protein in the precipitate. When lysate was incubated with both [^{14}C]tyrosine and [^3H]phenylalanine, or with both [^{14}C]lysine and [^3H]phenylalanine, both ^{14}C and ^3H were found in the TCA precipitates and the effect of temperature was equally apparent as with [^3H]phenylalanine alone. In the accompanying studies, incorporation of ^3H was measured and, inasmuch as this is influenced by pool sizes of endogenous phenylalanine, whole cells and lysate cannot be compared.

PROTEIN BREAKDOWN: In two studies, spermatids were incubated at 34°C with [^3H]phenylalanine for 30 min, washed twice with a cold phosphate-buffered saline medium and

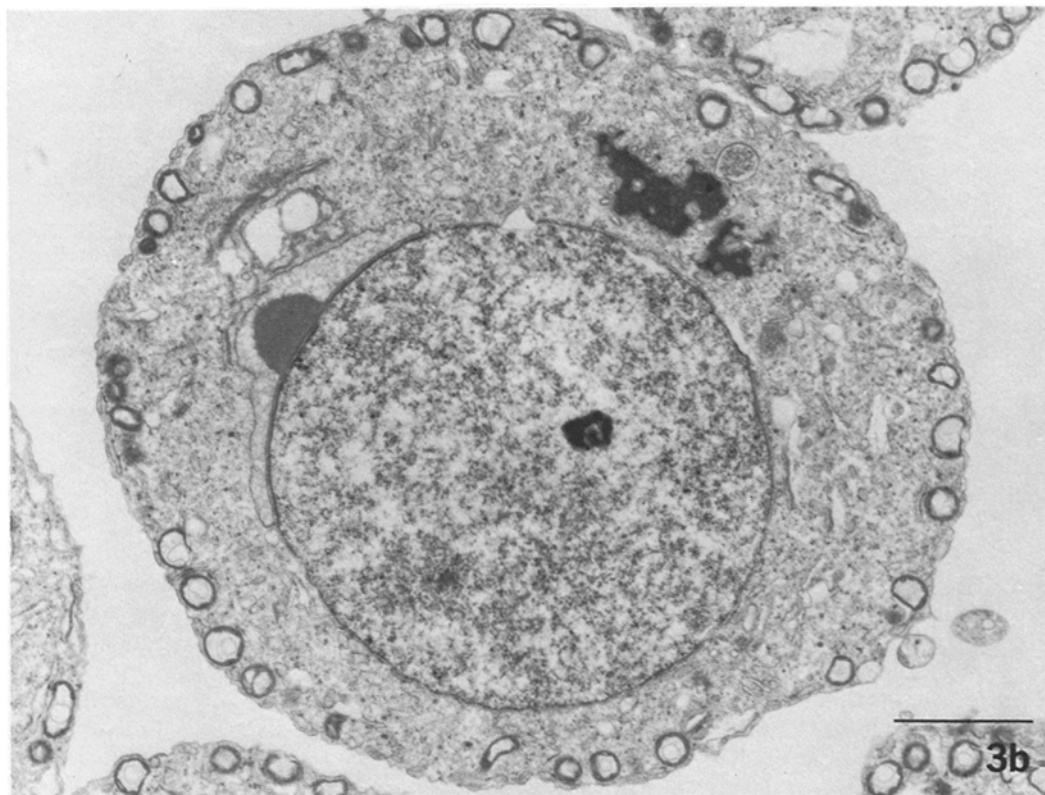
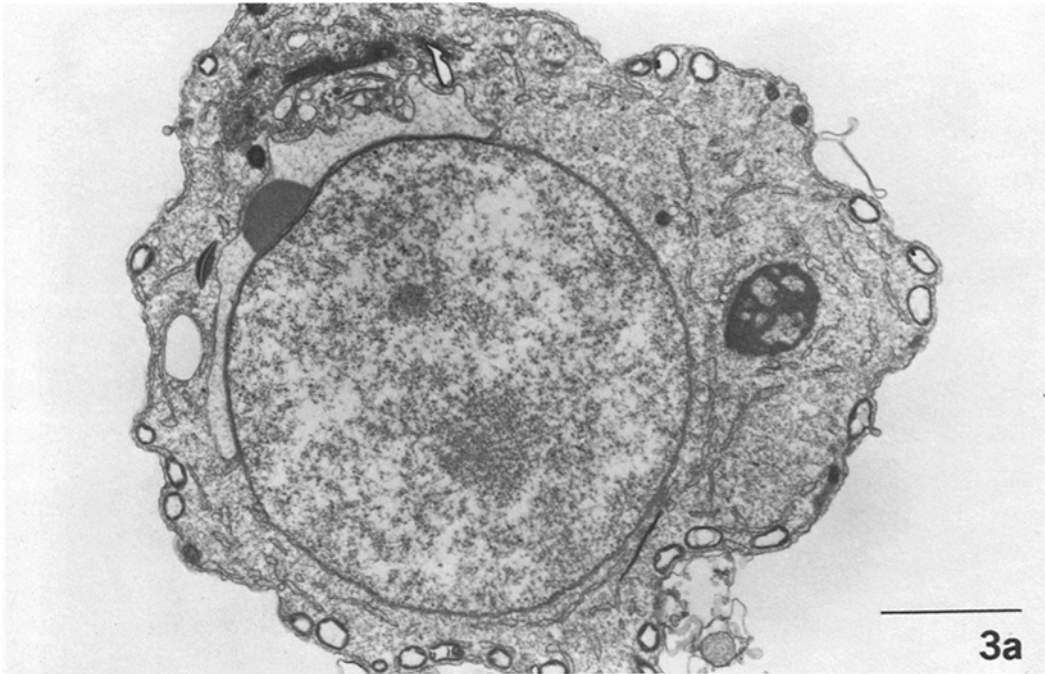


FIGURE 3 Electron micrographs of spermatids in step 6 or 7 of spermiogenesis. The Golgi apparatus is adjacent to the forming acrosome, which at this stage is beginning to form a cap over the nucleus. A densely staining chromatoid body is also present. The mitochondria are of a conformation typical of germ cells at this stage of development and are arranged in a characteristic array near the plasma membrane. (a) Spermatid from a cell suspension fixed immediately after dissociation of the testis with collagenase. The cell is structurally similar to cells *in situ* within the testes. (b) Spermatid from an immature spermatid fraction prepared by centrifugal elutriation. The cell is typical of cells from this fraction. It is slightly larger and more spherical than the cells fixed immediately after dissociation (Fig. 3a). Swelling of the nuclear envelope and cytoplasmic and nuclear vacuolization are also seen. The plasma membrane is intact. Bars, 2 μm ; $\times 9100$.

TABLE I
Metabolism of Immature Spermatids

Additions	Oxygen consumption	Lactate production
	nmol/min/10 ⁶ cells	
None	0.43 ± 0.07 (9)	<0.01 (5)
Glucose (10 mM)	0.77 ± 0.08	0.300 ± 0.010

Immature spermatids were incubated in phosphate-buffered saline at 34°C for 1 h for lactate. Oxygen consumption and lactate production were measured on separate batches of cells. The methods used are described in Materials and Methods. The values presented are mean and standard error of mean. The number of observations is given in the parentheses.

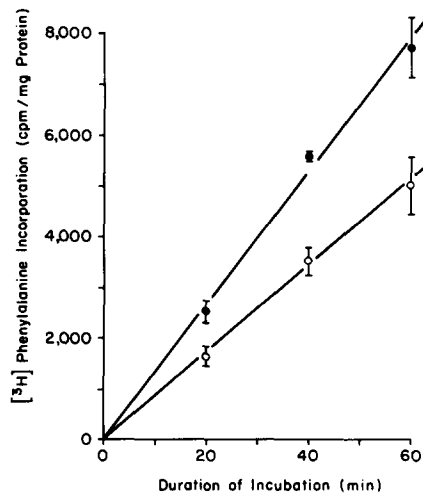


FIGURE 4 Immature spermatids were prepared and incubated with [³H]phenylalanine with and without glucose (10 mM) for the times shown. Incorporation of [³H]phenylalanine into spermatid protein was measured (Materials and Methods): (○) without glucose, (●) with glucose. The values shown represent means ± SD from three determinations.

transferred to the medium without amino acid. One batch was then incubated at 34°C and the other at 38°C. Samples of cells were taken at various times during the next 45 min, and incorporation of ³H into TCA precipitate was measured. The decline of ³H content in the precipitates with time was little and the same at both 34° and 38°C (data not shown).

DISCUSSION

The electron microscopic appearance of the immature spermatid fraction described here demonstrates that the cells are undoubtedly immature spermatids since these cells show the morphologi-

cal characteristics of immature spermatids in whole testis seen by electron microscopy. Light microscopy reveals that the cell fraction contains

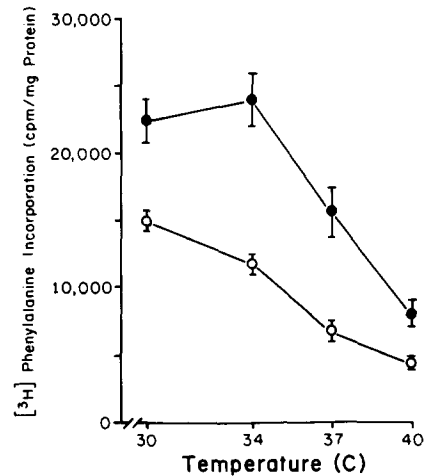


FIGURE 5 Incorporation of [³H]phenylalanine into testicular cell suspensions. Cells were dispersed by treatment with collagenase, and the enzyme was removed by washing (Materials and Methods). Suspended testicular cells were then incubated with [³H]phenylalanine to determine incorporation of amino acids into protein. The values shown represent means ± SD from five observations. 10 mM glucose (●), no glucose (○).

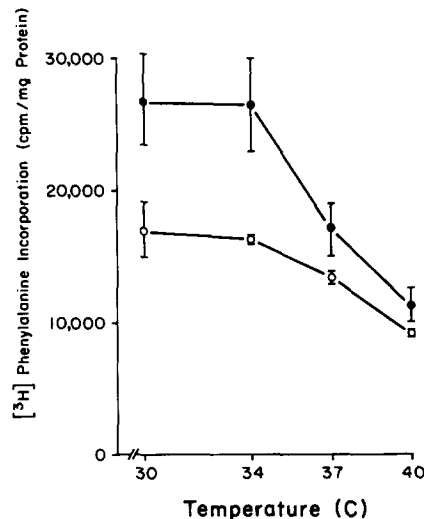


FIGURE 6 Effect of temperature on incorporation of [³H]phenylalanine into protein by purified immature spermatids. The cells were prepared by centrifugal elutriation and incubated with [³H]phenylalanine in separate incubators at the temperature shown. Incorporation of amino acid into protein was measured after incubation. The values shown represent means ± SD from four observations. 10 mM glucose (●), no glucose (○).

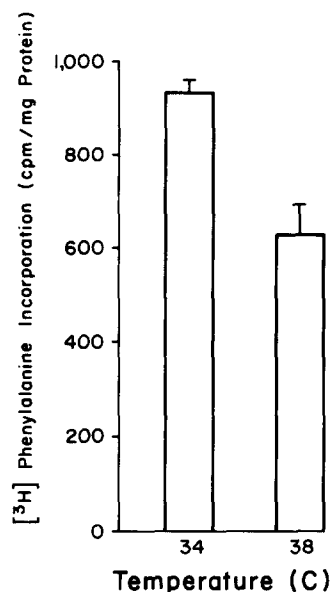


FIGURE 7 Incorporation of [³H]phenylalanine into spermatid protein by a subcellular preparation from immature spermatids. The cells isolated from adult rat testes by centrifugal elutriation were broken by lysis and incubated as described in Materials and Methods at the two temperatures shown. Incorporation of [³H]phenylalanine into protein was measured as described under Materials and Methods. The bars represent means with standard deviation from three observations.

>80% immature spermatids. Moreover, the method used to prepare these cells provides sufficient immature spermatids from four rat testes for biochemical studies. Electron microscopy further confirms exclusion of trypan blue in demonstrating that the cell membrane is intact. One feature of the cells which develops during preparation is the appearance of vacuoles. Similar vacuoles in spermatids have been described by other workers (1). A large number of modifications of the preparation procedure have failed to improve this feature of the cells. Vacuoles are not seen when spermatids in whole-cell suspensions are examined immediately after enzymatic dispersion, but they do develop when such suspensions are kept at 4°C for 2 h. It would therefore appear that vacuoles do not result from centrifugal elutriation per se. Until conditions for keeping the cells are improved or until the time required for separation can be considerably shortened, the best preparations of these cells are likely to show nuclear vacuoles and this feature should be kept in mind when the behavior of the cells *in vitro* is considered.

The immature spermatids are biochemically viable as far as metabolism of glucose, lactate production, and protein biosynthesis are concerned. The term "protein biosynthesis" is used because our findings apply to at least three amino acids and because incorporation into peptide bonds was demonstrated by methods already reported (reference 14 and Materials and Methods). In the subcellular system, the process was characterized in greater detail to demonstrate that this system was optimal and that two amino acids were incorporated simultaneously, excluding the possibility that we were merely measuring synthesis of polyphenylalanine.

Protein biosynthesis in suspensions of testicular cells shows, at least qualitatively, the same responses to temperature and glucose as that in whole testis (Fig. 5 and reference 2). The process of separating testicular cells from the whole organ has, to this extent at least, not altered the cells. Moreover, immature spermatids show similar responses to glucose and temperature as unseparated cells (Fig. 5), in keeping with earlier suggestions that spermatids are largely responsible for the temperature sensitivity and response to glucose seen with whole testis (4, 13). It is interesting to notice that the specific activity of protein synthesized by spermatids is very close to that of mixed cell suspensions. Because incorporation of [³H]phenylalanine is a complex process influenced by transport, pool sizes, and peptide bond formation, this similarity cannot be explained by our data but is presumably coincidental.

To explore the mechanism(s) by which body temperature inhibits protein synthesis by immature spermatids, we have made use of the limited number of cells available by confining our studies to two temperatures, namely 34° and 38°C, and developing a subcellular system capable of incorporating [³H]phenylalanine into protein. We assume that these temperatures can be related to scrotal and body temperatures, respectively (2, 7, 16). With this approach, it appears that body temperature inhibits protein biosynthesis in a subcellular system provided with excess ATP and GTP. Evidently, the inhibitory effect of body temperature includes an effect(s) on the intracellular steps of protein synthesis and cannot be accounted for by changes in amino acid transport alone. Moreover, although glucose greatly decreases the effect of temperature on protein synthesis by whole testis and in spite of the fact that glucose increases testicular levels of ATP (15),

body temperature inhibits spermatid protein synthesis when excess sources of energy are available to the subcellular system. The subcellular system also excludes an effect on amino acid transport as the only factor involved in decreased incorporation at 38° relative to 34°C. However, transport may be important in responses observed in whole cells. Inasmuch as incorporation of labeled amino acids is being measured, it is not possible to compare incorporation by lysate with that by whole cells because pool sizes of unlabeled endogenous amino acids are likely to be different.

Lee and Fritz (9) have shown that body temperature promotes release of lysosomal enzymes in mammalian germ cells and that such enzymes could destroy proteins. When cells were preincubated with [³H]phenylalanine at 34°C and then incubated without amino acid at 34° or 38°C, decrease with time of ³H in TCA precipitates was the same at 34° as at 38°C. This result shows that the effect of incubation at 38°C cannot be entirely explained by increased release of lysosomal enzymes with increased proteolysis as opposed to decreased synthesis at 38°C. However, this does not mean that such enzymes are without effect *in vivo*, because proteins may be incompletely degraded and still be precipitable by TCA. The nature of the inhibitory effect of body temperature on the incorporation of amino acids into protein is at present under investigation in this laboratory.

This investigation was supported by research grant GB43493 from the National Science Foundation and a Biochemical Research Grant from the University of Florida, College of Medicine.

Received for publication 1 September 1977, and in revised form 26 May 1978.

REFERENCES

1. BARCELONA, W. T., and M. L. MEISTRICH. 1977. Ultrastructural integrity of mouse testicular cells separated by velocity sedimentation. *J. Reprod. Fertil.* **50**:61-68.
2. DAVIS, J. R., and R. N. MORRIS. 1963. Effect of glucose on incorporation of L-lysine-U-¹⁴C into testicular proteins. *Am. J. Physiol.* **205**:833-836.
3. DAVIS, J. R., C. F. FIRLIT, and M. A. HOLLINGER. 1963. Effect of temperature on incorporation of L-lysine-U-C¹⁴ into testicular proteins. *Am. J. Physiol.* **204**:696-698.
4. DAVIS, J. R., and C. F. FIRLET. 1965. Effect of glucose on uptake of L-lysine-³H in cells of the seminiferous epithelium. *Am. J. Physiol.* **209**:425-432.
5. GRABSKE, R. J., S. LAKE, B. R. GLEDLILL, and M. L. MEISTRICH. 1975. Centrifugal elutriation: separation of spermatogenic cells on the basis of sedimentation velocity. *J. Cell. Physiol.* **86**:177-190.
6. GRIFFITHS, J. 1893. The structural changes in the testicle of the dog when it is replaced within the abdominal cavity. *J. Anat. Physiol.* **27**:483-500.
7. HALL, P. F. 1965. Influence of temperature upon the biosynthesis of testosterone by rabbit testis *in vitro*. *Endocrinology.* **76**:396-402.
8. HOHORST, H. 1965. *Methods of Enzymatic Analysis*. H. Bergmeyer, Editor. Verlag Chemie Weinheim, 266.
9. LEE, L. P. K., and I. B. FRITZ. 1972. Studies on spermatogenesis in rats. V. Increased thermal lability of lysosomes from testicular germinal cells and its possible relationship to impairments in spermatogenesis in cryptorchidism. *J. Biol. Chem.* **247**:7956-7961.
10. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-245.
11. NAKAMURA, M., and P. F. HALL. 1976. Inhibition by 5-thio-D-glycopyranose of protein biosynthesis *in vitro* in spermatids from rat testis. *Biochim. Biophys. Acta.* **447**:474-583.
12. MEANS, A. R., and P. F. HALL. 1968. Protein biosynthesis in the testis. II. Role of ATP in stimulation by glucose. *Endocrinology.* **83**:86-96.
13. MEANS, A. R., and P. F. HALL. 1968. Protein biosynthesis in the testis. I. Comparison between stimulation by FSH and glucose. *Endocrinology.* **82**:597-602.
14. MEANS, A. R., and P. F. HALL. 1967. Effect of FSH on protein biosynthesis in testis of immature rat. *Endocrinology.* **81**:1151-1160, 1967.
15. MEANS, A. R., and P. F. HALL. 1969. Protein biosynthesis in the testis. III. Dual effect of glucose. *Endocrinology.* **84**:285-297.
16. MOORE, C. R. 1922. Cryptorchidism experimentally produced. *Anat. Rec.* **24**:383.
17. ROMRELL, L. J., A. R. BELLVE, and D. W. FAWCETT. 1976. Separation of mouse spermatogenic cells by sedimentation velocity. *Dev. Biol.* **49**:119-131.
18. SHIKITA, M., and P. F. HALL. 1974. The Stoichiometry of the conversion and hydroxycholesterols to pregnenolone catalyzed by adrenal cytochrome P-450. *Proc. Natl. Acad. Sci. U.S.A.* **71**:1441-1445.