DEVELOPMENTAL BIOLOGY 87, 37–45 (1981)

Developmental Program of PGK-1 and PGK-2 Isozymes in Spermatogenic Cells of the Mouse: Specific Activities and Rates of Synthesis

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Received December 22, 1980; accepted in revised form April 20, 1981

The specific activities and synthesis of the ubiquitous isozyme, PGK-1, and the testis-specific isozyme, PGK-2, have been quantitated and localized in spermatogenic cells of the mouse. There is a fivefold increase in total PGK specific activity between immature and adult testes which begins at approximately 30 days of age, coincident with the appearance of late-middle stage spermatids. The increase in total PGK is entirely due to the appearance and increase of the PGK-2 isozyme. Rates of PGK synthesis were measured by labeling testicular cells *in vitro* with [^{3}H]leucine and purifying the PGK isozymes. When total testicular cells were examined, PGK-2 synthesis was detectable after 22 days of age at very low levels and increased in older testes to a level of 0.5% of total protein synthesis. PGK-1 synthesis remained relatively constant at all ages at a level 100-fold lower (0.005%). Testicular cells were separated into highly enriched fractions of particular spermatogenic stages by centrifugal elutriation. The PGK-1 synthesis rates were, again, very low and not significantly different between the various spermatogenic stages. PGK-2 synthesis was low to nondetectable in pachytene spermatocytes, increased to 0.07% in early spermatids and represented 0.7% of total protein synthesis in late spermatids. This increased rate of PGK-2 synthesis appears to require an increase in the amount of PGK-2 mRNA in late spermatids, cells in which no active RNA synthesis is detectable.

INTRODUCTION

The differentiative process of spermatogenesis involves extensive biochemical and morphological alterations which must require regulated gene expression. Much of this differentiation occurs after meiosis during the haploid spermatid stages, which in the mouse last approximately 14 days. Most genetic evidence argues against haploid gene expression in spermatogenesis (Beatty, 1970; Brock, 1977; Moreno *et al.*, 1980) although *t*-allele transmission ratio distortion has only been explainable as due to postmeiotic gene action (Erickson, 1978; Gluecksohn-Waelsch, 1972; Hammerberg and Klein, 1975). We are studying the phosphoglycerate kinase (PGK) isozymes of the testis as a model for the regulation of gene expression during spermatogenesis, in particular as a means to investigate postmeiotic gene expression.

Two isozymes of PGK have been described in mammals, PGK-1 and PGK-2. PGK-1 is a ubiquitous enzyme, found in all tissues examined; its structural locus (*Pgk-1*) is located on the *X*-chromosome (Chen *et al.*, 1971; Cooper *et al.*, 1971; Kozak *et al.*, 1973). PGK-2 is found only in the testes and sperm of most mammals (VandeBerg *et al.*, 1973) and is autosomally inherited (Eicher *et al.*, 1978; VandeBerg *et al.*, 1976, 1980). PGK-2 activity is first found in homogenates of developing immature testes when postmeiotic cells begin to appear (VandeBerg *et al.*, 1976; Erickson *et al.*, 1979). Specific immunofluorescence for PGK-2 is localized exclusively to postmeiotic germ cells of the testis (Kramer, 1981a). The intensity of the fluorescence increases in cells at later stages of differentiation, indicating that the concentration of PGK-2 increases as cells progress toward becoming mature sperm. These data suggest that PGK-2 may be an enzyme that is exclusively synthesized in the haploid, postmeiotic cells of spermatogenesis. Thus, elucidating the mechanisms which control PGK-2 expression may provide information on the control of gene expression in postmeiotic cells.

In this paper we have measured the levels of both PGK isozymes and their rates of synthesis throughout the course of spermatogenesis. The results indicate that PGK-2 synthesis is detectable only in postmeiotic cells and that the rate of synthesis increases in later postmeiotic stages.

MATERIALS AND METHODS

Materials. Agarose (low-M_r) and all acrylamide gel reagents were obtained from Bio-Rad. 2-Napthol-6,8-disulfonic acid dipotassium salt (NDA)[1] was from East-

[1] Abbreviations used: BSA, bovine serum albumin; DTT, dithiothreitol; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; NDA, 2-napthol-6,8-disulfonic acid dipotassium salt; PBS, Dulbecco's phosphate-buffered saline; PGK, phosphoglycerate kinase; TCA, trichloroacetic acid.

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man Kodak. Collagenase (136 units/mg), trypsin (9000 units/mg), phosphate-buffered saline (PBS), and all cell culture materials were obtained from GIBCO. [3,4,5-^{3}H]Leucine (118.3 Ci/mmole) was from New England Nuclear. Agarose-hexane-adenosine 5'-triphosphate (AGATP Type 4) linked via the ribose hydroxyls was obtained from P-L Biochemicals. Bovine serum albumin (BSA), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), deoxyribonuclease I (DN-CL), all substrates and cofactors, and all other chemicals were obtained from Sigma. Crystalline soybean trypsin inhibitor was purchased from Worthington.

Animals. CD-1 male mice purchased from Charles River or random bred from their stock were used for all experiments. They were maintained under a 14-hr-light, 10-hr-dark cycle with 3:1, pine:cedar bedding. The CD-1 mice in our colony segregate two allelic forms of PGK-2: PGK-2A and PGK-2B. These allozymes are separable by both agarose gel electrophoresis and two-dimensional gel electrophoresis. No differences in enzyme specific activity or rates of synthesis have been detected between PGK-2A and PGK-2B. We have therefore pooled the data for 2A and 2B and refer to it simply as PGK-2.

Determination of PGK specific activities. Animals were sacrificed by cervical dislocation, the testes removed, decapsulated, and homogenized in 5 vol of 10 m*M* Tris-HCl pH 7.4, 5 m*M* magnesium chloride, 1 m*M* DTT in a glass-Teflon homogenizer. Samples were centrifuged at 15,000*g* for 15 min and PGK activity was immediately assayed.

PGK activity was determined in a total reaction volume of 0.52 ml by adding 0.01 ml of appropriately diluted sample to 0.5 ml of reaction mix containing: 1 m*M* NAD, 1 m*M* glyceraldehyde-3-phosphoric acid, 0.45 unit of glyceraldehyde 3-phosphate dehydrogenase, 5 m*M* $MgCl_2$, 50 m*M* Tris-HCl (pH 7.5), 6 m*M* K_2HPO_4, 120 m*M* potassium acetate, 0.02% BSA. The mixture was allowed to equilibrate to 37°C, 0.01 ml of 0.09 *M* ADP was added, and the increase in absorbance at 340 nm was recorded. Total protein concentrations of samples were determined by the method of Lowry *et al.* (1951) using crystalline BSA as the standard. PGK specific activity was calculated as micromoles NAD reduced per minute per milligram protein.

PGK isozyme gel electrophoresis. PGK-1 and PGK-2 were separated by horizontal electrophoresis in gels (8 × 14 × 0.3 cm) consisting of 1% agarose, 5 m*M* citric acid, 5 m*M* histidine (pH 7.4) on a Pharmacia flat bed apparatus (FPB-3000). The electrode buffer was 0.1 *M* Tris, 0.03 *M* citric acid (pH 7.4). All samples were diluted such that 0.04 unit of total PGK activity were loaded per well. The apparatus was cooled with a circulating ice-water bath and electrophoresis was carried out at 12 W constant power per gel for 2–3 hr. The gel was then overlayed with a piece of Whatman No. 1 filter paper soaked in 0.1 *M* Tris-HCl (pH 8.0), 9 m*M* $MgCl_2$, 5.4 m*M* EDTA, 4 m*M* 3-phosphoglyceric acid, 0.84 m*M* NADH, 4 m*M* ATP, 15 units/ml glyceraldehyde 3-phosphate dehydrogenase, and incubated at 37°C for 15–20 min. The filter paper was removed and the gel was photographed under uv illumination with a yellow filter and Polaroid Type 55 P/N film. The negatives were fixed for 2–3 min in 18% sodium sulfite.

To determine the relative activities of PGK-1 and PGK-2, the negatives from the isozyme gel photographs were scanned on a Densicord Recording Electrophoresis Densitometer. Peak areas corresponding to PGK-1 and PGK-2 were determined by cutting out and weighing the peaks. The ratio of PGK-2 to PGK-1 activities was estimated from the ratio of the peak areas. A band of activity corresponding to 0.004 iu was easily detected.

Preparation of testicular cell suspensions. Testes were decapsulated and the intact tubules incubated at 32°C in 0.1% trypsin, 0.025% EDTA in PBS (pH 7.2) for 10 min. The solution was gently streamed over the tubules with a Pasteur pipet to remove interstitial cells and separate the tubules. The tubules were washed twice with PBS, chopped into 2- to 3-mm fragments with a razor blade and incubated in 0.21 mg/ml trypsin, 0.04 mg/ml deoxyribonuclease I in Hank's salts (pH 7.2) for 20 min at 32°C in a shaking water bath (120 cycles/min). The suspension was made 10% in newborn calf serum to inhibit trypsin, filtered through an 80 μm nylon screen, cooled, and centrifuged at 200*g* for 10 min at 4°C. The cells were washed once in Hank's with 0.1 mg/ml soybean trypsin inhibitor and once more with Hank's alone. Aliquots were removed for cell counting with a hemocytometer, viability determination by trypan blue exclusion, and *in vitro* labeling.

Separation of testicular cells by centrifugal elutriation. Separation of testicular cells into fractions at different stages of spermatogenesis was performed with a Beckman JE-6 elutriator rotor driven by a Beckman J21C centrifuge essentially as described by Grabske *et al.* (1975). The separation buffer used was 0.5% BSA, 1 m*M* NDA in PBS (pH 7.2) cooled to 4°C. Cells were loaded into the separation chamber and fractions collected as follows: Fraction 1 (2020 rpm, 19.2 ml/min), Fraction 2 (1500 rpm, 17.4 ml/min). The rotor was then stopped and all remaining cells were washed from the chamber. The Fraction 1 cells were then reloaded and fractionated as follows: Fraction 4 (5000 rpm, 11.2 ml/min), Fraction 5 (2020 rpm, 8.4 ml/min), Fraction 6 (2020 rpm, 16.5 ml/min), Fraction 7 (rotor stopped, >20 ml/min). Cells were collected from the fractions by pelleting (200*g*, 10 min) and washed twice in Hank's. Aliquots were removed for identification of cell types

by staining, cell counting, viability determinations, and *in vitro* labeling.

Preparation of spermatids from X-irradiated testes. Spermatids were prepared from X-irradiated animals as described by Geremia *et al.* (1978). Animals were exposed to 300 R of total body X-irradiation on Day 1 to kill spermatogonia, followed by 100 R on Days 7 and 13. This depletes the testis of nearly all germ cells except spermatids. On Day 14 the testes were removed, decapsulated, and incubated with 1 mg/ml collagenase in Hank's at 32°C for 15 min in a shaking water bath to remove interstitial cells. The tubules were washed three times in Hank's and pipetted up and down several times with a Pasteur pipet to release spermatids from the tubules. Tubule fragments were allowed to settle, the supernatant was removed, filtered, and centrifuged (200*g*, 10 min). The cells were washed twice in Hank's, aliquots were removed for cellular identification, cell counting, viability determination, and *in vitro* labeling.

Preparation of spermatocytes from immature testes. A homogeneous fraction of spermatocytes was prepared from the testes of 18 to 19-day-old mice. A single cell suspension was prepared as described above and centrifugal elutriation was performed collecting only two fractions: Fraction 1 (2020 rpm, 19.2 ml/min), Fraction 2 (1500 rpm, 19.2 ml/min). Fraction 2 is highly enriched for spermatocytes and was processed as described above.

Cellular identification. To identify the cell types present in various fractions, aliquots of cells were smeared on microscope slides, air dried, and fixed in Bouin's for 1 hr. The slides were washed in tap water for 30 min and stained with periodic acid–Schiff (PAS) and counterstained with Mayer's hematoxylin. Cells were identified following the criteria of Meistrich *et al.* (1973).

In vitro labeling of cell suspensions. Washed cells were resuspended at 15×10^6 cells/ml in Dulbecco's modified Eagle's medium with Hank's salts supplemented with 10 m*M* Hepes, lacking cold leucine but containing 50 μCi/ml [^{3}H]leucine. The cells were incubated at 32°C in air for 1 hr and incorporation was stopped by placing the culture tubes in ice and adding 3-4 vol of 0.5 mg/ml leucine in Hank's salts. The cells were centrifuged (200*g*, 10 min) and washed twice more in the cold leucine-containing Hank's. The cell pellets were lysed by adding 0.5 ml of 50 m*M* Tris-HCl (pH 7.5), 5 m*M* $MgSO_4$, 1 m*M* DTT, vortexing, and performing one freeze–thaw. Samples were centrifuged for 10 min in a Beckman Microfuge B before further use.

Purification of PGK and determination of rates of synthesis. The method for purification of PGK from radiolabeled cell lysates has been described and validated elsewhere (Kramer, 1981b). The cell lysate (0.1–0.5 ml) is passed through a 25-μl ATP–agarose column (AGATP Type 4; P-L Biochemicals) equilibrated with column buffer (50 m*M* Tris-HCl, pH 7.5, 5 m*M* $MgSO_4$). The column is successively washed with 0.15 ml column buffer, 0.05 ml column buffer containing 0.7 *M* NaCl, and finally 0.2 ml column buffer. Typically, 85–95% of the starting PGK activity is retained on the column and PGK represents approximately 80% of the total protein retained. The affinity column bed with bound PGK is then directly transferred with lysis buffer (9 *M* urea, 2% NP-40, 0.1 *M* DTT, 2.8% total Ampholines as a 3:1:3 mixture of pH 7–9:5–7:3.5–10) to the first-dimension isoelectric focusing gel of a modified two-dimensional O'Farrell type system (O'Farrell, 1975). After completion of two-dimensional gel electrophoresis, the Coomassie-stained spots corresponding to PGK-1 and PGK-2 are separately excised, digested, and counted. Rates of syntheses are calculated as the percentage of total TCA-precipitable counts per minute recovered in the purified PGK-1 or PGK-2 spot. These values are adjusted to account for the recovery of PGK activity during purification and for the efficiency of scintillation counting, as determined by the internal standards method.

RESULTS

As the immature testis develops, cells at later stages of the spermatogenic series sequentially appear and increase in number. The coincident appearance or increase of a specific protein at the same time as a specific spermatogenic cell type suggests the association of the protein with that cell type. The total PGK specific activity in testis homogenates from mice of various ages is presented in Fig. 1a. Beginning at approximately 30 days of age there is a marked increase in PGK specific activity, with near adult levels being reached by 50 days of age. The specific activity of PGK in adult testes is approximately five times greater than that in testes from animals less than 30 days old. At 30 days of age late-middle stage spermatids first appear in the testis, suggesting that these cells have an increased PGK specific activity. Chronological age is not the best indicator of the developmental stage of the testis, however, due to the multitude of biological factors which affect growth and development rates.

We have chosen testis weight as another indicator of the developmental stage of the testis. The relationship between PGK specific activity and paired testes weight is presented in Fig. 1b. PGK activity remains constant until a paired testes weight of 0.14 g is attained, at which point a dramatic increase in PGK is evident. Maximal levels of PGK are attained when the paired testes exceed 0.20 g in weight. A plot of paired testes weight versus age is presented in Fig. 1c. From

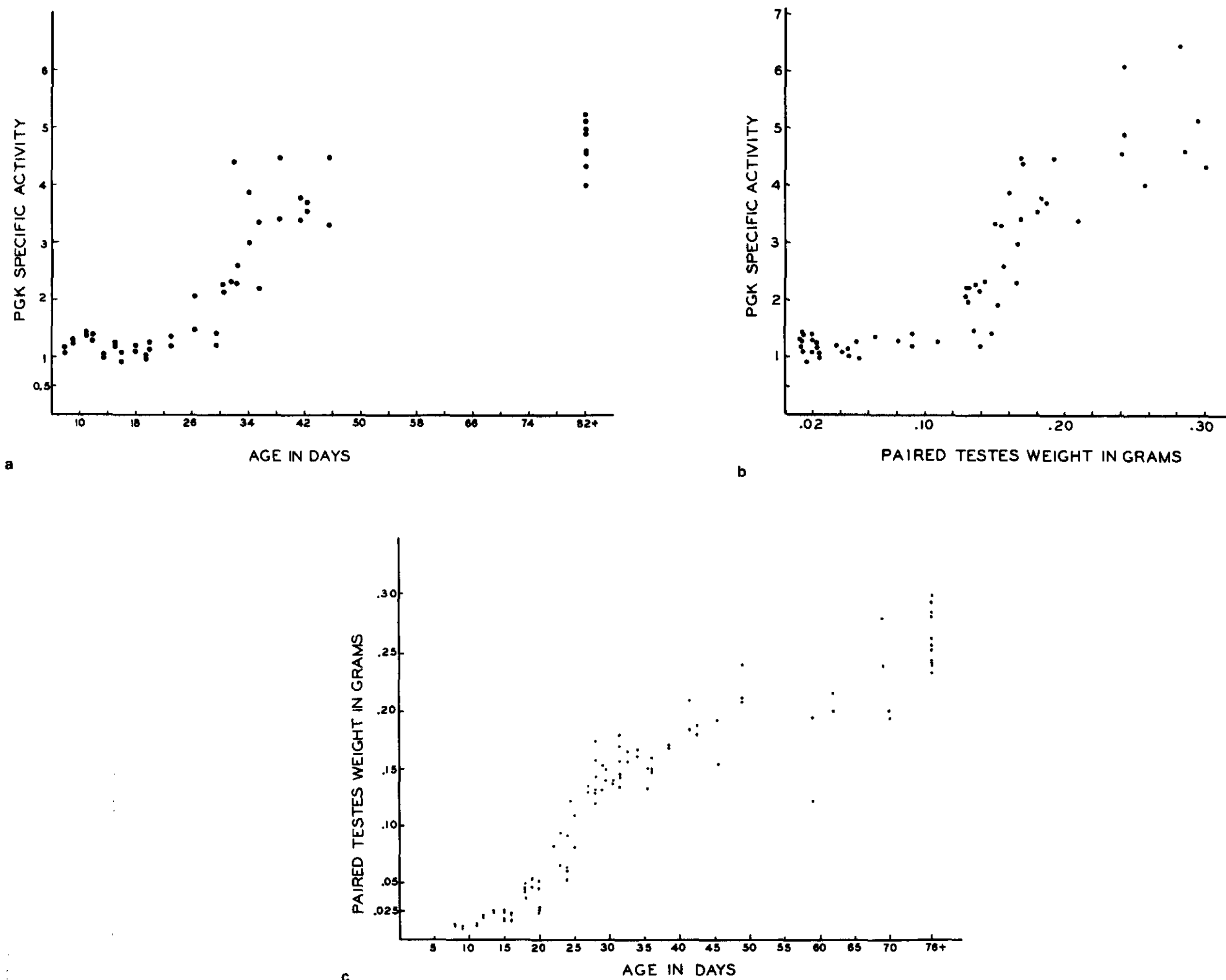

FIG. 1. PGK specific activities in developing testes. (a) PGK specific activity (μmole NAD reduced/min/mg protein) in testicular homogenates from mice of various ages. (b) PGK specific activity in testes homogenates plotted against the weight of the paired testes. Both testes of a single animal were trimmed of all external fat and tissue; the tunica was not removed. (c) Testes weight as a function of animal age. Each point represents a single animal, except for animals less than 20 days of age (less than 0.05 g paired testes weight), where testes were pooled. All PGK assays were done in duplicate and the means are presented.

this figure, a paired testes weight of 0.14 g corresponds to an age of between 28 and 32 days. Thus, at a point in the development of the testis corresponding to 0.14 g paired weight, or approximately 30 days of age, an increase in PGK specific activity is initiated which results in a fivefold increase in the mature testis. This increase is coincident with the appearance of late-middle stage spermatids, implying that they have a much higher PGK specific activity than earlier spermatogenic cells.

In order to determine the relative contributions of the two isozymes, PKG-1 and PGK-2, to the total PGK activity, the isozymes were separated by agarose gel electrophoresis. Homogenates from testes of different ages were diluted such that the same amount of total PGK activity (0.04 unit) was analyzed from each sample. Photographic negatives of the isozyme gels were scanned on a densitometer. In Fig. 2 the ratios of PGK-2/PGK-1 activities during development are presented. Beginning at 26 days, the ratio increases from zero to a final value of 4.5 in the mature testis. The fivefold increase in total PGK, demonstrated in Figs. 1a and 1b, can be entirely accounted for by this increase in PGK-2. The final ratio of 4.5 indicates that PGK-2 represents approximately 80% of the total PGK activity in the mature testis. Enzyme immunoinactivation studies with an antiserum specific for PGK-2 have also demonstrated that PGK-2 accounts for approximately 80% of the total PGK in mature testes (Erickson *et al.*, 1979). PGK-2 activity is first detectable on agarose gels in testes from 22 to 23-day-old animals, but only when 2.5–3.0 times the usual amount of total PGK activity is run and then only as a very faint band. The intensity of this band is approximately equivalent to the PGK-2 band

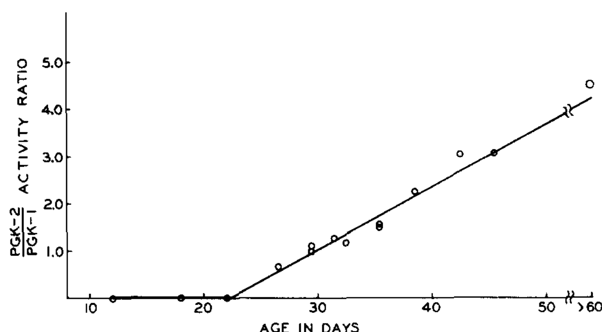

FIG. 2. Relative activities of PGK-1 and PGK-2 in total testes homogenates from mice of various ages. Homogenates were electrophoresed on agarose gels and stained as described under Materials and Methods. Photographic negatives of the stained gels were scanned on a densitometer. The activity ratios were estimated as the ratios of the peak areas from the densitometer scan. All points represent single determinations except for the value at >60 days of age, which is the mean of the values for three separate animals. The line is the best fit linear regression for the points between 26 and 45 days ($r^2 = 0.95$).

of a mature testis homogenate diluted 32-fold more than usual. Thus, the PGK-2 activity in 22 to 23-day testes is $<1\%$ of the PGK-2 level in mature testes and $<0.8\%$ of the total PGK activity in 22 to 23-day testes.

These data indicate that the specific activity of PGK-1 remains relatively constant at 1 μmole/min/mg protein throughout the development of the testis. PGK-2 first appears at 22–23 days at very low levels <0.008); at 30 days PGK-2 begins to increase rapidly until the adult value of 4 units/mg protein is attained. The first appearance of PGK-2 at 22 days is coincident with the appearance of early spermatids which contain very low levels of PGK-2. When late-middle stage spermatids appear, at approximately 30 days, a large increase in total PGK activity is observed which is entirely due to the PGK-2 isozyme, implying that these and later stage spermatids have increasing PGK-2 specific activities.

As a first step towards understanding how the levels of the PGK isozymes are regulated in the testis, we have studied their rates of synthesis. The rates of synthesis of both PGK-1 and PGK-2 were determined utilizing a miniature PGK purification method involving affinity chromatography and two-dimensional gel electrophoresis, which we have previously characterized in detail (Kramer, 1981b). This method provides high recoveries (80–95%) and clearly separates the isozymes, PGK-1 and PGK-2. Testicular cell suspensions (3.7–28 $\times 10^6$ total cells at a concentration of 15×10^6 cells/ml) were cultured for 1 hr with [^{3}H]leucine and aliquots of the resulting cell lysates (84,000–2,000,000 TCA-precipitable cpm) were subjected to the purification scheme. Duplicate determinations for each individual animal were carried out with the exception that before 30 days of age, testes were pooled from several animals. Incorporation of [^{3}H]leucine into TCA-precipitable material was linear with time for at least 90 min. Cell viabilities, both before and after labeling, ranged from 93 to 97%.

The rates of PGK-2 synthesis (percentage total TCA-precipitable counts per minute) for total testes cells from testes of various ages are presented in Fig. 3. Little or no PGK-2 synthesis was detectable in testes of animals from 15 to 22 days of age, while significant amounts of PGK-2 synthesis were detected in testes from animals 24 days and older. An abrupt rise in rate of synthesis, from 0.028 to 0.11%, is evident after 30 days of age, coincident with the increase in PGK specific activity also seen at that time. The rate of PGK-2 synthesis remains relatively constant from 30 to 54 days of age at about 0.1% of total protein synthesis. After 54 days, there may be a further increase in the mean rate of PGK-2 synthesis, however, there is considerable

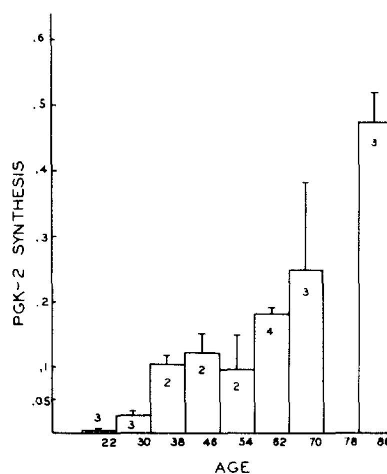

FIG. 3. Rates of PGK-2 synthesis in total testicular cell suspensions from mice of various ages. Cells were labeled for 1 hr *in vitro* with 50 μCi/ml [^{3}H]leucine. PGK-2 was purified, and synthetic rates were calculated as the percentage of total TCA-precipitable cpm after correcting for enzyme recovery and counting efficiency. Duplicate determinations were performed for each cell preparation and the mean values were used. Results were pooled into age groups of 8 days and the number of independent testicular cell suspensions analyzed is indicated for each age group. The height of each column represents the mean for that age group and the bars represent 1 SEM.

variability between individual animals at these ages. Four of the six animals examined at age 70 or 85 days had rates of synthesis between 0.41 and 0.56%; however, the other two animals had rates of synthesis of approximately 0.1%. It is not clear whether there is a consistent increase in PGK-2 synthesis at this time in development.

The PGK-1 synthetic rates determined in testes of various ages are presented in Fig. 4. The rates of PGK-1 synthesis are very low, averaging about 0.005%, and no significant differences are apparent between 15 and 86 days of age. This is consistent with the PGK-1 specific activity data which show that PGK-1 activity remains relatively constant throughout this period. With the present methods it would be difficult to detect small changes in this low rate of PGK-1 synthesis. However, the 50- to 100-fold difference between rates of synthesis of PGK-2 (0.3–0.5%) and PGK-1 (0.005%) is clearly significant.

In order to determine which cell types are responsible for the synthesis of each PGK isozyme, cell suspensions were fractionated by elutrial centrifugation. Three highly enriched fractions were obtained, with purities similar to those previously reported (Erickson *et al.*, 1980a). Fraction 2 was 85% pachytene spermatocytes; Fraction 6 was 80% early spermatids; Fraction 5 was 40% middle and late stage spermatids and 50% cytoplasts derived from late spermatids (Romrell *et al.*, 1976). These cell fractions were labeled in culture and PGK was purified as previously described. The results of two independent cell elutriations, each assayed in duplicate, are presented in Fig. 5. The pachytene spermatocytes (Fraction 2) exhibit very little or no PGK-2 synthesis. The amount observed can be accounted for by the small degree of contamination of this fraction with early spermatids. In early spermatids (Fraction 6) PGK-2 represents approximately 0.07% of total protein synthesis and in middle and late spermatids (Fraction 5) it has increased to 0.7% of total protein synthesis. PGK-1 synthesis in these fractions is approximately 100-fold lower than PGK-2 synthesis and there are no significant differences between the fractions.

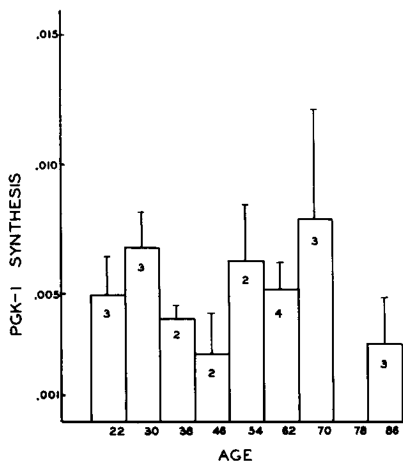

FIG. 4. Rates of PGK-1 synthesis in total testicular cell suspensions from mice of various ages. Determinations were made as described in Fig. 3 except that PGK-1 was purified. Note difference in scale as compared to Fig. 3.

To confirm the results obtained with cells fractionated by elutrial centrifugation, spermatids and spermatocytes were prepared by alternate methods. Spermatids were obtained from mice which had undergone three X-irradiation treatments to deplete their testes of all other germ cells. The resulting cell suspensions contained approximately 90% spermatids of all stages; the major contaminant was Sertoli cells. The PGK synthetic rates determined from six separate spermatid preparations assayed in duplicate are presented in Fig. 5 as Fraction X. The PGK-2 synthetic rate is 0.5%, a value between those for the early and the late spermatids prepared by elutriation. This is as expected since the Fraction X spermatid preparations contain a mixture of early and late spermatids. The rate of synthesis of PGK-1 for these cells is only 2% of the rate for PGK-2. The value observed in these cells is a little higher than previously observed (0.009%); this may be due to the contamination with Sertoli cells which appear to have a relatively high concentration of PGK-1 (Kramer, 1981a).

Finally, spermatocytes were prepared from the testes of ten 18- to 19-day-old mice, as described under Materials and Methods. Few, if any, spermatids have appeared in testes of this age. The resulting cell suspension, consisting of approximately 80% spermatocytes, was assayed for PGK synthesis (Fig 5, Fraction S). The rate of PGK-2 synthesis was very low, and did not differ from the rate determined with spermatocytes isolated from mature testes. PGK-1 synthesis was in the same range as previously described (0.01%).

As spermatogenic cells differentiate from the spermatocyte stage to the mature sperm there is a decrease in both cell size (Lam *et al.* 1970) and rate of protein synthesis (Monesi, 1965a). Therefore, the values for specific activity (units/per milligram protein) and rates of synthesis (percentage of total protein synthesis) could increase if the amount of PGK per cell and its absolute rate of synthesis did not change during this period. When the data on PGK activity and PGK-2 synthesis from the elutriator fractions of adult testes are examined, the specific activity (units per milligram) of PGK is about 4.5-fold greater in late spermatids (6.7 units/mg) than in early spermatids (1.5 units/mg), in agreement with the developmental data described above. When expressed as PGK activity per cell, there is still a 2.5-fold difference between early spermatids (0.05 unit/10^6 cells) and late spermatids (0.13 unit/10^6 cells).

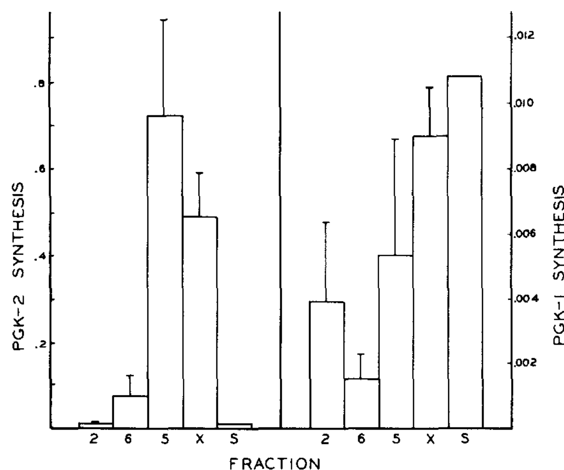

FIG. 5. Rates of PGK-1 (right) and PGK-2 (left) synthesis in fractionated testicular cells. Testicular cells fractionated into distinct spermatogenic stages were labeled for 1 hr with [^{3}H]leucine, PGK-1 and PGK-2 were purified, and rates of synthesis were calculated as the percentage of total TCA-precipitable radioactivity recovered in purified PGK after correcting for enzyme recovery during purification and counting efficiency. Duplicate determinations were performed for each cell preparation. Fractions 2, 6, and 5 were obtained by centrifugal elutriation; the mean and standard error for two experiments are presented. Fraction 2, pachytene spermatocytes; Fraction 6, early spermatids; Fraction 5, late spermatids. Fraction X designates spermatids prepared from X-irradiated mice; the mean and standard error for six experiments are presented. Fraction S represents spermatocytes prepared from 18-day-old mice. Note difference in scales between PGK-1 and PGK-2.

This 2.5-fold increase in the amount of PGK could result from increased synthesis of PGK, decreased degradation of PGK, or a combination of both.

When PGK-2 synthesis is expressed as percentage of total protein synthesis, there is a 10-fold increase in the mean rate of PGK-2 synthesis during the transition from early spermatids (0.07%) to late spermatids (0.72%). In these experiments, the total incorporation of [^{3}H]leucine into TCA-precipitable material was 4.7-fold higher in early spermatids (105,700 cpm/10^6 cells) than in late spermatids (22,400 cpm/10^6 cells). Thus, after accounting for the difference in total synthesis, there is still a two-fold increase in PGK-2 synthesis between early spermatids and late spermatids. This analysis of the data assumes that these cell types do not differ substantially with respect to the specific activity of the leucine pool available for protein synthesis. This appears to be the case for lysine pools in rat testicular cells (Brock *et al.*, 1980). It may be relevant to note that in mouse spermatogenic cells the uridine pool appears to increase in later stage cells (Geremia *et al.*, 1977). If the same were true for amino acid pools, the increase in absolute rate of synthesis of PGK-2 in late spermatids would be even larger. In summary, even though total protein synthesis is reduced in late spermatids, the per-cell synthesis of PGK-2 appears to increase.

DISCUSSION

The data presented in this and the accompanying paper can be summarized as follows. There is a gradual fivefold increase in the total PGK specific activity of the developing testis which begins when late-middle stage spermatids first appear. The increase in PGK activity is entirely accounted for by the first appearance and increase of the testis specific isozyme PGK-2. This increase is paralleled by an increasing rate of PGK-2 synthesis in total testis cells. Both the specific activity and rate of synthesis of the ubiquitous isozyme, PGK-1, are relatively constant as the testis develops. In the mature testis, the rate of PGK-2 synthesis is 50–100 times greater than that of PGK-1. PGK-2 is exclusively localized to the postmeiotic germ cells of the testis. The concentration of PGK-2 in these cells increases as they progress through the differentiative stages to mature sperm. The synthesis of PGK-2 also follows this pattern, since PGK-2 synthesis increases during the transition between early and late spermatids. PGK-1 synthesis, on the other hand, is at very low levels in germ cells and is not detectably different in different cell stages—the small amounts of synthesis apparently seen in some cell types may be due to contaminating Sertoli or other cells.

The elevated PGK-2 synthesis in late as compared to early stage spermatids is evident as an increased percentage of total protein synthesis and there appears also to be an increased number of PGK-2 molecules synthesized per cell per unit time. Such an increased absolute rate of PGK-2 synthesis requires that there is either an increase in the amount of PGK-2 mRNA and/or an increased translation efficiency. Autoradiographic studies with [^{3}H]uridine have failed to detect RNA synthesis in middle and late spermatids (Stages

9-16) (Monesi, 1965a, 1971; Moore, 1971; Kierszenbaum and Tres, 1975; Söderström and Parvinen, 1976). Biochemical studies of RNA purified from fractionated spermatogenic cells have also failed to detect RNA synthesis in middle and late spermatids (Geremia *et al.*, 1977, 1978; Erickson *et al.*, 1980a). The lack of RNA synthesis during the period of increase in PGK-2 synthesis raises the question of how this increase is mediated.

A very low level of mRNA synthesis in middle and late spermatids, which would be difficult to demonstrate experimentally, might account for the increase in PGK-2 synthesis seen in these cells. Alternatively, an increase in active PGK-2 mRNA may result from the utilization of "stored" mRNA. It has been demonstrated that a large amount of RNA synthesized in pachytene spermatocytes is stably maintained within the cell throughout the entire differentiation of the spermatid, for up to 17 days (Monesi, 1965a; Geremia *et al.*, 1977). When total cellular RNA prepared from spermatocytes, early spermatids, and middle to late spermatids was translated *in vitro* in a rabbit reticulocyte system, PGK-2 synthesis was detected only with the RNA prepared from middle to late spermatids (Erickson *et al.*, 1980b). This suggests that the amount of translatable PGK-2 mRNA is greatly increased in these cells. This does not conclusively differentiate between new mRNA synthesis and "stored" mRNA, however, because the "stored" mRNA may exist in an untranslatable form prior to the detected increase in PGK-2 synthesis.

The time and rates of synthesis for two other testis-specific proteins, lactate dehydrogenase-X (LDH-X) and protamine-like histone (PLH), have been studied. LDH-X is first detectable in pachytene spermatocytes; the specific activity increases in later subsequent cell types but the amount of LDH-X per cell actually declines in these later cells. The synthesis of LDH-X is highest in pachytene spermatocytes and declines progressively in more mature cells (Meistrich *et al.*, 1977). Thus, it was proposed that all of the LDH-X mRNA is synthesized in the spermatocyte and there is a slow decline in its level as the cell matures. This indicates that although LDH-X and PGK-2 are both testis-specific isozymes, their patterns of regulation during spermatogenesis are quite different. Alternatively, the appearance and synthesis of PLH does not occur until late-middle stage spermatids (Monesi, 1965a; Lam and Bruce, 1971; Bellvé *et al.*, 1975) and translatable PLH mRNA is only detectable at this time (Erickson *et al.*, 1980b). This corresponds to the time of increase in PGK-2 synthesis and suggests that the synthesis or activation of mRNA for both PGK-2 and PLH may be coordinately regulated. Further study of this possibility may reveal whether there is coordinate control of multiple genes during spermatogenesis.

The specific function of PGK-2 in spermatogenic cells is not known. While the testis-specific isozyme LDH-X has unique enzymatic properties (Zinkham *et al.*, 1964; Goldberg, 1972; Burgos *et al.*, 1979) which led to the proposal that it fulfills a unique metabolic function in spermatozoa (Storey and Kayne, 1977), the enzymatic properties of PGK-1 and PGK-2 are indistinguishable (Pegoraro and Lee, 1978; Lee *et al.*, 1980). Currently no special metabolic function for PGK-2 is known. One hypothesis to explain the appearance of PGK-2 is based on the possibility that the X-chromosome in males is inactivated at meiosis during spermatogenesis (Monesi, 1965b; Kofman-Alfaro and Chandley, 1970; Lifschytz and Lindsley, 1974). The *Pgk-1* locus is X-linked and therefore would be inactivated at meiosis. The autosomal *Pgk-2* locus might then be activated to provide a source of PGK activity for the postmeiotic germ cells. If this were so, the level of PGK-1 mRNA and the rate of PGK-1 synthesis would be expected to decline after meiosis. We have not detected such a decline in PGK-1 synthesis, but because PGK-1 synthesis is so low we cannot conclude with assurance that such a decline does not occur. A strong test of this hypothesis will require the use of cDNA probes to quantitate PGK-1 mRNA synthesis and concentration.

The PGK isozymes provide a potentially useful system for studying the mechanisms that control postmeiotic gene expression and for testing the hypothesis of X-chromosome inactivation during spermatogenesis. The possibility that PGK-2 synthesis is controlled by a "stored" mRNA, and the existence of PGK-1 mRNA synthesis after meiosis, may be directly evaluated when specific cDNA probes are produced.

This work was supported by Genetics Training Grant GM7544 and Research Grant HD 11738 to R. P. Erickson. This work was submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in the Horace H. Rackham School of Graduate Studies at the University of Michigan. We would like to thank Mrs. Rena Jones for excellent secretarial assistance.

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