Changes in Acidic Chromatin Proteins during the Hormone-dependent Development of Rat Testis and Epididymis*

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SUMMARY
The acidic chromatin proteins of rat testis and epididymis have been characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in terms of their composition and relative rates of synthesis and phosphorylation in vitro. The proteins extracted from testis were resolved reproducibly into 23 specific bands. At 3 days of age, the high molecular weight Bands 1 through 5 were not detectable, but with the formation of spermatagonia and spermatocytes these bands accumulated, there was a marked increase in the rates of synthesis of Bands 1 through 9, and a marked decrease in the net rates of phosphorylation of the majority of these proteins was observed. Among the epididymal proteins, 22 specific bands were observed during development. Cell proliferation and differentiation in the epididymis at puberty were characterized by a marked decrease in Bands 1, 2, and 20, and emergence of Bands 8 through 17 as the predominant components. This process was associated with a marked increase in the rates of synthesis and a reduced net rate of phosphorylation of the majority of the epididymal acidic chromatin proteins. Changes in rates of phosphorylation in these experiments related primarily to phosphorylation of serine and threonine residues in preformed polypeptide chains, and did not reflect alterations in the specific activity of the [(γ-32P)ATP pools. Developmental patterns of change in acidic chromatin proteins in testis were found to be dependent upon pituitary gonadotropic hormones, while those of epididymis were dependent upon testosterone, as demonstrated by hormone replacement experiments with hypophysectomized rats. These results demonstrate patterns of change in formation and composition in populations of acidic chromatin proteins at well defined transitions in cell differentiation in testis and epididymis and are consistent with the hypothesis that these proteins may participate in the regulation of gene expression during the development of these organs.

Spermatogenesis in higher animals involves a complex process of hormone-dependent cell differentiation in which each cell type is characterized by the presence of cell-specific protein markers. In the testis of the rat, this process consists of a highly synchronized sequence of molecular events, so that the concentrations of specific proteins may be correlated with the appearance of new cell types on a developmental time scale (1-6). During puberty and shortly before spermatozoa appear in the seminiferous tubules, the epididymis undergoes androgen-dependent growth with differentiation of ciliated and clear cells which participate in the subsequent transport and further maturation of the male gametes (7-9). The molecular determinants of these orderly developmental changes are largely unknown.

Recently, considerable evidence has been assembled to support the hypothesis that gene regulation and variable gene expression during cell differentiation may be influenced by acidic chromatin proteins. The properties of these proteins meet several criteria anticipated for potential gene regulators: they have organ and species specificity; they exhibit great heterogeneity in their electrophoretic and immunological properties; they are more actively synthesized in active and proliferating cells; they bind more specifically to the DNA of their origin, and modify the sequence of hybridizable RNA that are transcribed on reconstituted chromatin (10-14). Qualitative changes in populations of acidic chromatin proteins have been reported during cell differentiation in the slime mold Physarum polycephalum (15, 16), in sea urchin embryos (17-19), in mouse mammary cells (20-22), and in avian erythrocytes (23). In the studies reported here, the acidic proteins of chromatin extracted from rat testis and epididymis cells have been characterized in terms of their composition and relative rates of synthesis and phosphorylation. The results demonstrate patterns of change in nuclear protein composition and synthesis which correlate with well defined transitions in hormone-dependent cell differentiation in these organs.

EXPERIMENTAL PROCEDURE

Materials

Animals—Male Sprague-Dawley rats were used for all experiments. Animals were subjected to surgical hypophysectomy at age 21 or 26 days, and the completeness of hypophysectomy in each animal was determined by measurement of adrenal weights and body weight, and by examination of the sella turcica at the time of sacrifice.

Chemicals and Hormones—L-[2,3-3H]Aspartic acid (N) (specific activity, 26 Ci per mmole), L-[4,5-3H]leucine (N) (specific activity, 35 Ci per mmole [5-3H]uridine [22 Ci per mmole], and L-[3H]serine

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(specific activity, 30 Ci per mmole) were purchased from New England Nuclear, Boston, Mass. Carrier-free [32P]orthophosphate was obtained from International Chemical & Nuclear Corp., Irvine, Calif. Testosterone cypionate (200 mg per ml of ethanol) was purchased from Nutritional Biochemicals, Cleveland, Ohio, and was redissolved in water. Urea purchased from Mann Research Laboratories, New York. Ovine prolactin, follicle-stimulating hormone NIH-LH-S17, oxine follicle-stimulating hormone NIH-FSH-59, oxine prolactin NIH-P-59, and ovine growth hormone NIH-GH-88 were gifts from the Endocrinology Study Section, National Institute of Arthritis and Metabolic Diseases.

Methods

Preparation of Tissues—All tissues were chilled on ice throughout the procedures. Rats were killed by cervical dislocation and the testes and epididymides were rapidly excised via the abdominal route. The tunica albuginea of the testis was left intact, and the epididymides were dissected free of the testes and trimmed of fat and connective tissue. For radioactivity-labeling experiments, whole testes or minced epididymides were cultured in 24-well dishes (Linbro, Associate, Bethesda, Maryland) at 32° in a humidified atmosphere of 95% air and 5% CO2. The unsealed vials were shaken at 120 cpm. Isotopic precursors were present in the medium in the following concentrations: [3H]leucine, 25 μCi per ml; [3H]serine, 10 μCi per ml; [3H]orthophosphate, 50 μCi per ml. The time of incubation in all in vitro labeling experiments was 3 hours unless stated otherwise. The incubated epididymides were subsequently combined with sufficient "carrier" epididymal tissue to ensure adequate recovery of nuclei and chromatin protein.

Preparation of Nuclei—Nuclei were isolated and purified by a modification of a previously described method (24). Tissues were homogenized in 0.25 M sucrose (1:6, w/v) containing 0.05 M Tris-HCl (pH 7.5), 0.0025 M KCl, and 0.005 M MgCl2 in a motor-driven Teflon-glass Potter-Elvehjem homogenizer. Prior to homogenization, the epididymides were minced in homogenizing medium and then washed to remove any spermatozoa. The final homogenate was mixed with an equal volume of 0.88 M sucrose containing 0.01 M sodium phosphate and 0.15 M sodium citrate. A glass homogenizer fitted with a motor-driven Teflon pestle was used, and the homogenate was centrifuged each time at 19,000 × g, for 10 min. The final purified chromatin pellet was resuspended in 2.0 ml of 0.1 M NaCl, 0.05 M sodium phosphate (pH 6.0) and extracted of histones by the method of Spelsberg et al. (27). The chromatin suspension was then dispersed by homogenization and was centrifuged at 100,000 × g, for 30 hours to form a pellet of dehistonized chromatin.

Polycrylamide Gel Electrophoresis—The acidic proteins derived from dehistonized chromatin preparations were analyzed by polycrylamide gel electrophoresis by a modification of the method of Teng et al. (12), as previously described (20). Disaggregation of the dehistonized chromatin pellet was achieved by homogenization in 0.01 M sodium phosphate buffer (pH 7.4) containing 6 M urea, 0.1% sodium dodecyl sulfate and 0.1 M 2-mercaptoethanol. The mixture was dialyzed for 24 to 36 hours at 4° against the same buffer. Centrifugation at 25,000 × g, for 15 min yielded a clarified supernatant which was then subjected to electrophoresis. The proportion of total protein recovered in the supernatant fluid ranged between 91 and 96% in all preparations. (Protein samples (100 μg in 200 μl in all experiments) were subjected to electrophoresis in duplicate using 6 μl per gel for 5 to 7 hours at 27°. The polycrylamide gels contained 7.5% acrylamide, 0.2% bisacrylamide, 0.1 M sodium phosphate (pH 7.4), 0.1% sodium dodecyl sulfate, 6 M urea, and 0.05 M 2-mercaptoethanol. The electrophoresis, staining of the gels, and sectioning of the gels were carried out as previously described (20). Gel absorbancy was determined by scanning in 10 ml of toluene scintillation fluid at 40% efficiency for 4H and with an average counting error of less than ±1% (S.E.). The average variability of radioactivity in corresponding gel slices from replicate electrophoresis was ±10% (S.E.).

Other Analytical Methods—32P-Iabeled acidic chromatin proteins were hydrolyzed for 2 N HCl in a boiling water bath for 16 hours. HCl was removed under vacuum, and the hydrolysate was subjected to paper electrophoresis at 4° on Whatman No. 1 paper strips (5 × 20 cm) using 8% formic acid as the electrophoresis buffer. Electrophoresis of samples of phosphoserine and phosphohistidine standards was conducted for 10 hours at a constant voltage of 400 volts and an average current of 3.0 ma per strip. Ninhydrin-staining spots were cut out and counted in toluene scintillation fluid. DNA was determined by the diphenylamine method of Burton (28). Protein was measured by the method of Lowry et al. (29). Amino acid compositions were determined using a Beckman 120 amino acid analyzer, as previously described (20).

Hormone Treatments—All hormones were prepared and stored as previously described (1), and were administered as subcutaneous injections. Polypeptide hormones were injected every 12 hours in the following amounts: luteinizing hormone, 50 μg; follicle-stimulating hormone, 10 μg; growth hormone, 25 μg; prolactin, 50 μg. L-Triiodothyronine (0.10 μg), cortisone acetate (1.0 mg), and testosterone propionate (3.0 mg) were administered once daily.

Chemical Properties of Acidic Chromatin Protein Preparations—The relative amino acid compositions of acidic chromatin protein preparations from rat testis and epididymis are shown in Table I. The relative content of aspartic and glutamic acids greatly exceeds that of the basic amino acids, lysine, arginine, and histidine.

| Table 1 |
|---------------------------------|
| Relative amino acid compositions of acidic chromatin proteins derived from rat testis or epididymis (50 days of age) |
|---------------------------------|
| Values are expressed as moles per 100 moles of total amino acids recovered, and have not been corrected for hydrolytic losses or amide content. |

| Amino acid           | Testis | Epididymis |
|----------------------|--------|------------|
| Aspartic acid        | 7.71   | 8.55       |
| Glutamic acid        | 12.79  | 11.93      |
| Threonine            | 5.54   | 5.85       |
| Serine               | 5.25   | 4.71       |
| Proline              | 7.63   | 7.49       |
| Glycine              | 12.37  | 16.27      |
| Alanine              | 6.73   | 8.42       |
| Valine               | 5.18   | 5.79       |
| Methionine           | 1.48   | 1.91       |
| Isoleucine           | 3.58   | 4.27       |
| Leucine              | 8.50   | 6.75       |
| Tyrosine             | 4.71   | 4.26       |
| Phenylalanine        | 4.71   | 2.50       |
| Histidine            | 1.89   | 2.13       |
| Lysine               | 4.80   | 4.16       |
| Arginine             | 5.75   | 6.11       |
Electrophoresis of the proteins in polyacrylamide gels at pH values between 4 and 10 demonstrated that they have isoelectric points below pH 7.0, and were free of histone contamination. Extraction of the purified, $^{32}$P-labeled proteins with 10 volumes of ether removed less than 1% of the radioactivity, indicating an apparently low level of contamination by phospholipid. Following labeling of nuclear RNA with [3H]uridine (8.0 μCi per ml of medium for 30 min) 100 μg purified acidic chromatin protein contained insignificant radioactivity above background. These results indicate the relative purity and acidic nature of the isolated proteins.

RESULTS

Electrophoretic Characteristics of Acidic Chromatin Proteins of Rat Testis and Epididymis—The proteins of dehistonized chromatin derived from rat testis or epididymis were separated according to molecular size by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate. Subsequent staining with Amido black revealed highly heterogeneous banding patterns representing a broad range of molecular weights which were characteristic for the proteins derived from each tissue (Fig. 1). A highly reproducible pattern of 25 electrophoretic components was observed in the proteins derived from mature rat testis. The electrophoretic mobilities of many of the proteins derived from the epididymis differed significantly from those in the mixture derived from testis. The proteins in the electrophoretogram shown for the epididymis in Fig. 1 are numbered according to increasing electrophoretic mobility and taking into account the relative electrophoretic mobilities of all components which appear during development of the epididymis (see below). It was considered likely that the protein mixtures from testis and epididymis contained additional components to those resolved by this sodium dodecyl sulfate gel technique. However, the 25 components resolved from the testis protein and the 22 components identified in the epididymis system were chosen as markers for comparison at various stages of hormone-dependent development.

Estimates of the relative amounts of the individual protein components in the electrophoretic banding patterns for each of these tissues were made by absorbance scanning of the stained gels. The variability of individual absorbance peaks in replicate electrophoretic banding patterns was indicated by a standard error of the mean of ±6% or less (26). Fig. 2 shows the characteristic absorbance profiles of acidic chromatin proteins derived from testes of rats at 5 days, 24 days, and 44 days of age. At 5 days of age, the rat testis consists largely of Sertoli cells and very few disappearing gonocytes with occasional cells which are early spermatogonial cells. At this stage protein Bands 1 through 5, which are characteristic of the mature testis (Fig. 2C, 55 days of age), are not distinctly detectable. Bands 24 and 25 are the predominant protein species present, and several high molecular weight constituents which form a major component of the mature pattern are relatively decreased in amount. At 24 days of age, mature spermatogonia and spermatocytes have differentiated in the seminiferous tubules, and this stage is characterized by the appearance of distinct Bands 1, 2, and 5, with relative increase in Bands 6 through 9 (Fig. 2B). In the mature testis, all cellular components, including the spermatids and the final stage of differentiation, the spermatocytes, are present. The high molecular weight acidic chromatin protein Components 1 through 5 are distinct at this state (Fig. 2C), and Bands 6 through 14 represent dominant components of the electrophoretogram relative to Bands 24 and 25.

Similar observations have been made on the relative amounts of acidic chromatin proteins which are present at salient stages in the development of the rat epididymis, as shown in Fig. 3. At 20 days of age, the rat epididymis is in an undifferentiated state, and this state of development is associated with a characteristic profile of acidic chromatin protein (light tracing). At this stage, the high molecular weight Components 1 and 2 are distinct, and Band 20 represents a dominant component. In the mature rat epididymis (55 days of age), large ciliated cells have appeared in the head and distinct clear cells are present in the tail. This stage is associated with a predominance of Bands 8 through 17 (heavy tracing). In contrast to the earlier pattern, Bands 1 and 2 are undetectable and Band 20 is a minor constituent. At an intermediate stage of development, the midpubertal period of development at 33 days of age, an intermediate pattern is characteristic, as shown in Fig. 4. Bands 1 and 2, which were characteristic of the immature form, are less prominent, an intermediate amount of Band 20 is present, Band 22 is a major component, and numerous other constituents are present in relatively different proportions from those observed either at 20 or at 55 days of age. During this period, the head of the epididymis undergoes differentiation to form large ciliated epithelial cells. Band 12 in the absorbance profile of the whole organ is contributed primarily by the tail of the epididymis.
Synthesis of Acidic Chromatin Proteins in Vitro—Rat testes at various stages of development were incubated with [3H]leucine and [32P]orthophosphate, and the patterns of radioactivity labeling in acidic chromatin proteins were determined after electrophoresis of the extracted proteins in sodium dodecyl sulfate polyacrylamide gels. Under the conditions of in vitro labeling, the rate of incorporation of each isotope by testis and by epididymis (see below) was linear for 6 hours, as determined at hourly intervals. The pH of the medium remained constant during these incubations as indicated by visual examination of the phenol red indicator. Fig. 5 shows the electrophoretic radioactivity profiles in rat testes at 20 days, 35 days, or 60 days of age. In the 20-day testis, in which only Sertoli cells, spermatogonia, and early spermatocytes are present, there was a relatively lower rate of incorporation of [3H]leucine into acidic chromatin proteins of high molecular weight and a relatively higher rate of labeling of all bands with [32P]orthophosphate compared to subsequent stages of development. At 35 days of age, the spermatids have appeared, and the rate of incorporation of leucine was observed in the highest molecular weight components (bands 1 through 5). In the mature testis (60 days of age, Fig. 5C), the high and intermediate molecular weight
proteins were the predominant labeled species. In comparison with the 20-day testis (Fig. 5A), the testes at puberty (Fig. 5B) and at maturity (Fig. 5C) exhibited a markedly reduced net rate of phosphorylation of acidic chromatin proteins.

Changes in rates of synthesis and phosphorylation characteristic of specific stages in development were also evident from a similar analysis of the rat epididymis. The undifferentiated epididymis at 20 days (Fig. 6A) exhibited a relatively high net rate of phosphorylation, especially of the high molecular weight proteins (Bands 1 through 6), and a relatively lower net rate of synthesis of all the acidic chromatin proteins. During cell proliferation and differentiation, as exhibited at 35 days of age, a marked stimulation in the over-all rate of incorporation of \([3H]\)leucine was evident (Fig. 6B), and a relatively reduced net rate of phosphorylation of these proteins was observed. In the mature and fully differentiated rat epididymis (Fig. 6C), the rate of incorporation of \([3H]\)leucine was lower in comparison to the period of active development (Fig. 6B), and a similar level of the net rate of phosphorylation was observed but with a relatively higher net rate of incorporation in the higher molecular weight proteins (Bands 3 through 6). Table II summarizes the net rates of incorporation of \([3H]\)leucine and \([32P]\) into total acidic chromatin proteins of testis and epididymis at various stages of development in the rat.

The proteins of both tissues were remarkable for the unusually high rate of phosphorylation of threonine residues.

The relative rates of turnover of the acidic chromatin proteins in each of these tissues were estimated by chase experiments. As shown in Fig. 7, a 10 to 15% decrease in the over-all levels of retained \([3H]\)leucine was observed in testis during a 4-hour incubation in nonisotopic medium. In contrast, approximately 64% of the \([32P]\)orthophosphate label incorporated in testis during the initial 2-hour period turned over during the subsequent 4

![Fig. 5](image5.png)

**FIG. 5.** Electrophoretic radioactivity profiles of acidic chromatin proteins labeled with \([32P]\)orthophosphate and \([3H]\)leucine and extracted from rat testes at age 20 days (A), 35 days (B), or 60 days (C) of age. The position of the individual bands after electrophoresis was determined by comparison with the absorbance profiles of the stained proteins in each electrophoretogram. Each gel was sectioned transversely into individual 1-mm slices, and the front of each gel is at 6.1 cm.

![Fig. 6](image6.png)

**FIG. 6.** Electrophoretic radioactivity profiles of acidic chromatin proteins labeled with \([32P]\)orthophosphate and \([3H]\)leucine and extracted from rat epididymis at age 20 days (A), 35 days (B), or 60 days (C) of age. The position of the individual bands after electrophoresis was determined by comparison with the absorbance profiles of the stained proteins in each electrophoretogram. Each gel was sectioned transversely into individual 1-mm slices, and the front of each gel is at 6.3 cm.

**Table II**

Rates of incorporation of \([3H]\)leucine and \([32P]\) into total acidic chromatin proteins of testis and epididymis at various stages of development in the rat.

| Tissue     | Age | Radioactivity |
|------------|-----|---------------|
|            |     | \(\text{cpm/50 mg tissue/3 hrs}\) | \(\text{cpm/30 mg tissue/3 hrs}\) |
| Testis     | 20  | 12,000        | 14,000         |
|            | 35  | 19,100        | 4,100          |
|            | 60  | 15,400        | 6,400          |
| Epididymis | 20  | 3,400         | 14,700         |
|            | 35  | 10,900        | 6,600          |
|            | 60  | 4,700         | 7,400          |
hours of incubation. This turnover was evident throughout the electrophoretogram. Identical rates of turnover were obtained with acidic chromatin proteins derived from rat epididymis.

To determine whether the development-related changes in rates of phosphorylation of acidic chromatin proteins in rat testis and epididymis could relate to changes in the specific activity of the \(\gamma\)-ATP precursor, the specific activity of ATP in the nuclear and cytosolic fractions of each of these tissues was determined at different stages of development. As shown in Table IV, changes of only 5 to 10% were observed in the various tissues analyzed. These small changes in the specific activity of the radioactive precursor could not account for the changes of 100 to 400% observed in the results shown in Figs. 5, 6, 8, and 9.

To determine whether \([P^3P]\)orthophosphate was incorporated before or after completion of the acidic chromatin polypeptide chains, testes and epididymides were allowed to incorporate \([H^3]s\)erine and \([P^3P]\)orthophosphate for 1 or 2 hours in the presence or absence of cycloheximide (Table V). Although this inhibitor of polypeptide synthesis reduced incorporation of \([H]s\)erine into acidic chromatin proteins by 87 and 88% during the 1st hour of incubation, it did not significantly reduce the incorporation of \([P^3P]\)orthophosphate during this same period. During the 2nd hour, the inhibition of polypeptide synthesis was associated with marked inhibition of \([P^3P]\)orthophosphate incorporation. These results indicate that phosphorylation occurs following the completion of polypeptide synthesis.

### Table III

**Distribution of \(^{32}P\) in phosphoserine and phosphothreonine in acidic chromatin proteins of rat testis and epididymis**

| Tissue  | \(^{32}P\)Serine | \(^{32}P\)Threonine |
|---------|-----------------|-------------------|
| Testis  | 5760            | 6200              |
| Epididymis | 5520          | 8730              |

### Table IV

**Specific activity of ATP at various stages of development of rat testis and epididymis**

| Tissue       | Age  | \(^{32}P\)ATP |
|--------------|------|---------------|
| Testis       | days | (cpm/mg protein) |
| Whole cell  | 20   | 60,000        |
| 60           | 57,000 |
| Epididymis   | 34 (hypophysectomy at 26 days) | 62,000 |
| 20           | 76,000 |
| 35           | 80,000 |

### Table V

**Effect of cycloheximide on rate of incorporation of \([H]s\)erine and \([P^3P]\)orthophosphate into acidic chromatin proteins**

| Tissue     | Labeling period | \(^{32}P\)-protein | \(^{3}H\)-protein |
|------------|-----------------|-------------------|------------------|
| Testis     | hr              | cpm/100 μg protein | %         | cpm/100 μg protein | %         |
| Control    | 1               | 3,600             | 3,700          | 4,400                | 540                  | 88          |
| Cycloheximide | 2             | 7,100             | 4,200          | 5,700                | 660                  | 92          |
| Epididymis | 1               | 5,300             | 5,600          | 3,650                | 470                  | 87          |
| Control    | 2               | 10,700            | 8,400          | 7,200                | 590                  | 92          |

### Fig. 7

Electrophoretic radioactivity patterns of acidic chromatin proteins synthesized by 26-day-old rat testes. Intact testes were allowed to incorporate \([H]s\)erine (46 μCi per ml) and \([P^3P]\)orthophosphate (50 μCi per ml) during a 2-hour period of incubation. At the end of this labeling period, the testes were either analyzed for acidic chromatin proteins (A) or were washed in Medium 199 and transferred to nonisotopic medium for a 4-hour chase period. They were subsequently extracted of acidic chromatin proteins and subjected to electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate. The front is at 6.3 cm.
pletion of polypeptide chain synthesis. \(^{32}\)P labeling of acidic chromatin proteins by these techniques thus represents primary phosphorylation of preformed polypeptide chains as well as some phosphate exchange in acidic phosphoproteins of chromatin.

To determine whether or not any of the developmental patterns observed in the acidic chromatin proteins of testes or epididymis could relate to differential losses or proteolytic degradation of specific components during chromatin isolation, various mixtures of tissue homogenates were prepared. Homogenates of tissues labeled with \(^{3}H\)leucine and \(^{32}\)Porthophosphate were mixed with equal amounts of homogenates of the same organ at other stages of development, and acidic chromatin proteins were isolated and subjected to electrophoresis. Such mixtures exhibited no alteration of the radioactivity profile, there was no loss of high molecular weight bands, and the absorbance profiles generally represented additive effects of the individual preparations. The experimental design of these studies, however, does not rule out the possibility of different nuclear phosphatases which could act prior to the disruption of the nuclei in these preparations.

**Effects of Hormones on Synthesis of Acidic Chromatin Proteins—**

In order to determine whether the developmental changes in the synthesis of acidic chromatin proteins of rat testis and epididymis were dependent upon specific hormones, rats were subjected to surgical hypophysectomy and given replacement treatments with various combinations of hormones. Fig. 8A shows the electrophoretic radioactivity profile of nuclear acidic chromatin proteins of 35-day-old rat testis 9 days following hypophysectomy. In comparison with the proteins shown in Fig. 5, which were radiolabeled under identical conditions, the \(^{3}H\)leucine-labeling profile of the hypophysectomized rats closely resembled that of intact 20-day-old rats, and the developmental progression to labeling of two high molecular weight peaks (gel sections 2 through 10) was not observed. However, the age-related reduction in the \(^{32}\)P-labeling profile occurred in these hypophysectomized rats (cf. Fig. 5, A and B). Treatment of the hypophysectomized rats with luteinizing hormone and follicle-stimulating hormone was associated with the formation of the \(^{3}H\)leucine-labeling pattern shown in Fig. 8B. This profile closely resembled that of the intact 35-day-old rat (Fig. 5B), although the overall net rate of labeling with \(^{32}\)P was somewhat reduced. Treatment of other groups of rats with an additional combination of other pituitary-dependent hormones (growth hormone, prolactin, triiodothyronine, and cortisone) did not significantly alter the pattern shown in Fig. 8A, and treatment with these hormones in combination with luteinizing hormone and follicle-stimulating hormone did not significantly change the effect shown in Fig. 8B.

Similar studies were carried out in relation to the possible hormonal dependence of the developmental patterns of synthesis of acidic chromatin proteins in rat epididymis. Fig. 9A shows the labeling profiles of proteins derived from 34-day-old epididymides 13 days after hypophysectomy. In comparison with the proteins of Fig. 6A, which were radiolabeled under identical conditions, a slight reduction in rates of synthesis in the proteins in gel sections 1 through 40 was observed, and no progression to the pattern characteristic of the 34- or 35-day-old epididymis (Fig. 6B) was observed. Treatment of the hypophysectomized rats with testosterone caused a transformation of the labeling pattern, as shown in Fig. 9B. This pattern showing a relative increase in labeling with \(^{32}\)P and a relative decrease in labeling with \(^{3}H\)leucine, represented a radioactivity profile which was...
similar to the profile of epididymides of normal rats at 35 days of age (cf. Fig. 6B). Neither of the patterns shown in Fig. 9 was significantly altered by treating such animals concomitantly with growth hormone, prolactin, triiodothyronine, and cortisone. As shown in Table IV, these effects of hypophysectomy or hypophysectomy and testosterone treatment on net rates of phosphorylation could not be explained merely by a change in the specific activities of the $\gamma$-[32P]ATP precursor in these tissues.

**DISCUSSION**

These studies have characterized populations of acidic chromatin proteins during the development of the testis and epididymis of the rat. Electrophoretic analyses of these proteins have demonstrated their organ specificity (cf. Ref. 20) and have documented qualitative changes in multiple protein species at well characterized stages of development in each organ. Differentiation of spermatogonia and spermatocytes in the testis was associated with a marked increase in the rate of synthesis of certain high molecular weight proteins (Bands 1 through 9) and the accumulation of protein Bands 1 through 5, protein species which were not detectable in testes containing only Sertoli cells and gonocytes. This developmental transition was also marked by a decrease in the net rate of phosphorylation of the acidic chromatin proteins. A lower rate of phosphorylation was characteristic of the testis as the developing cell population acquired more highly specialized and less metabolically active cells (4, 5). Bands 24 and 25 of the testis appeared to be highly stable throughout development. They represented the predominant species in the testis at the Sertoli cell stage (5 days of age) and continued to represent a significant portion of the protein population in the fully developed testis, although the rates of incorporation of [3H]leucine and $^{32}$P into these species were low at all stages of development.

Differentiation of rat epididymal cells at puberty was also associated with an alteration in the relative content and rates of synthesis of specific acidic proteins of the chromatin of these cells. At 20 days of age, the epithelial cells are undifferentiated, and there is a characteristic electrophoretic profile of acidic chromatin proteins (Fig. 3) in which Band 20 is a dominant component and the high molecular weight Bands 1 and 2 are present. Differentiation of ciliated and clear cells occurs by Day 35, at which stage Band 22 increased relative to other components, and Band 20 was a relatively smaller component. During this pubertal period, there was a marked increase in the net rate of synthesis of the intermediate molecular weight proteins and a relative decline in their net rates of phosphorylation. This result is consistent with previous studies which have demonstrated increased rates of synthesis of acidic chromatin proteins in a variety of cell types during cell proliferation (13, 20, 31). Following this period of cell division and organ growth, there was a return to lower rates of protein synthesis. In the mature epididymis (age 55 days) in which cell differentiation was qualitatively complete, the composition of the acidic chromatin protein population differed from earlier stages of development in that Bands 20 and 22 were minor components, the minor Bands 1 and 2 of high molecular weight were no longer detectable, and Band 17 increased from the barely detectable level to a dominant peak.

The developmental changes in the relative rates of synthesis of acidic chromatin proteins in testis and epididymis were dependent upon hormones known to regulate cell differentiation in each organ. Hypophysectomy at 20 or 26 days of age in nearly every instance prevented the progression of the electrophoretic radioactivity profiles to those characteristic of the testis and epididymis of the intact 35-day-old rats. An exception to this general trend was the failure of hypophysectomy to alter the pattern of phosphorylation in the testis, and the explanation for this result is not apparent. Treatment of such hypophysectomized rats with luteinizing hormone and follicle-stimulating hormone was sufficient to induce continued spermatogenesis and to restore the normal relative rates of [3H]leucine incorporation into these proteins of the testis. Treatment of the hypophysectomized animals with testosterone was sufficient to induce pubertal growth and to restore the relative rates of synthesis and phosphorylation of the acidic chromatin proteins of the 35-day-old epididymis. However, the precise mechanisms by which stimulation with these specific hormones leads to altered rates of synthesis of specific acidic chromatin proteins in each organ remain to be elucidated. It has been reported that other hormones alter the rates of synthesis of acidic chromatin proteins in their respective target cells. Stimulation with cortisol (32), glucagon (33), and cyclic adenosine 3':5'-monophosphate (34) have been associated with hormone-specific increases in the rate of synthesis of specific acidic proteins of rat liver, and an increase in the net radioactivity labeling of non-histone nuclear proteins has been observed in rat liver after stimulation with insulin (35). Specific alterations in the phosphorylation or synthesis of acidic nuclear proteins have been reported for the actions of androgens on prostate cells (36), for 17b-estradiol on rat uterus (37), and for the action of ec dysone on insect chromosomes (38). Specific binding sites for steroid hormone-receptor complexes also exist in specific acidic chromatin proteins of the respective target cells of these hormones (39).

A considerable body of evidence relating to the properties of acidic chromatin proteins of a wide variety of eukaryotic cells supports the proposition that this class of proteins may serve as gene regulators (10-14). Previous studies from this laboratory have demonstrated that the synthesis of an apparently "new" group of high molecular weight proteins is initiated during chromosomal replication in mammary stem cells, and the formation of these acidic chromosomal proteins is a prerequisite for the production of phenotypically differentiated mammary alveolar cells and the induction of milk proteins (20-22). The present study on the acidic proteins of rat testis and epididymal chromatin demonstrates changes in this class of proteins which are consistent with the hypothesis that they may participate in gene regulation during cell differentiation in these organs. The proteins studied are chemically distinct from histones and other basic proteins previously characterized in the nuclei of spermatids (40, 41) and spermatozoa (41-43) of mammalian testes. The specific roles of the acidic chromatin proteins in potential protein-protein and protein-DNA interactions in epididymal and testis cells must await further studies utilizing purified protein components. Such studies are currently in progress in this laboratory.

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