

AUTORADIOGRAPHIC STUDY ON LOCALIZATION OF PROGESTIN TARGET CELLS IN THE CHICKEN TESTES

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Using an autoradiographic technique, the localization and concentration of progestin (P) in cell constituents of testes from the immature 25-to-70-day-old and mature 120-day-old chickens were investigated. After incubation of the frozen tissue sections with ^3H -Promegestone (R5020), a synthetic P, specific radioactivity, was found in the seminiferous epithelium and interstitium in all the animals in the presence or in the absence of various non-radioactive steroids, and the intensity of radioactivity differed with age: high in 25-, 70- and 120-day-old chickens, and low in 50-day-old chickens. Estrogen treatment enhanced the intensity of radioactivity in the cells at all ages. The concentration of radioactivity was higher in the seminiferous epithelium than in the interstitial cells. In the frozen tissue sections, it was not possible to identify the kind of cell taking up the high amount of radioactivity. These results indicate that the testes from chicken during their growing stages contain cell constituents that specifically bind P.

Key words: Progestin Target Cells, Chicken Testis, Autoradiography

INTRODUCTION

Testes from patients with testicular feminization have been shown to contain cytosol progestin (P) receptors¹⁾. During detailed investigations on P binding in testes from various animals including humans, monkeys, rats and chickens, Seiki et al.^{2~4)} found that only the testes from chicken contain cytosol P receptors, the molecular size of which is 8 S in mature animals and 4 S in immature animals. Administration of P to the chickens causes a decrease in testicular weight which is amplified by addition of estrogen (E) to P⁴⁾. The administration of P also causes several changes in metabolic activities within the testes: increase or decrease in the synthesis of protein, RNA and DNA, which is enhanced by the addition of E to P^{4~5)}. Most of those findings cannot, however, precisely explain which kinds of cells in the testes contain P receptors and whether the cell constituents containing P receptors are responsible for the changes in metabolic activities of the testis. Using an autoradio-

graphic technique the present study was carried out to detect the localization of P target cells interacting *in vitro* with diffusible radioactive P within the testis specimens of the chickens.

MATERIALS AND METHODS

Preparation of frozen tissue sections:

White Leghorns, 25, 50, 70 and 120 days old, were obtained from Shonan Chickyard, Isehara City, Japan. Animals of each age were divided into two groups; one was given i.m. injection of 2 μg 17 β -estradiol-3-benzoate (E₂B) / 0.1 ml sesame oil / 100 g b.w. once a day for 3 days, and the other was given i.m. injection of 0.1 ml sesame oil / 100 g b.w. once a day for 3 days. One day after the last injection, they were killed by decapitation. Testes were carefully extirpated, and stored frozen for 6~7 days at -80°C until use. The frozen tissues were sectioned at 5 μm at -20°C , mounted on clear glass slides, and air-dried. The dried tissue sections were fixed in absolute ethanol for 10 min at 4°C , washed by several changes

of phosphate-buffered saline (PBS) solution, pH 7.4, and then dried overnight at 4°C. Autoradiographic procedures:

^3H -Promegestone (R5020, s.a. 87 Ci/mmol), a potent synthetic P, and non-radioactive Promegestone were purchased from New England Nuclear (Boston, U.S.A.), and used without further purification. Progesterone, testosterone, estradiol-17 β and corticosterone were obtained from Sigma Chemical Company (St. Louis, U.S.A.). The mounted tissue sections were covered with 0.1 ml PBS solution containing 3 ng ^3H -R5020 in the presence or absence of 600 ng (200-fold excess dose) of unlabeled steroids such as R5020, progesterone, testosterone, estradiol-17 β or corticosterone. They were incubated for 2 hr at 20°C in a humidified box to avoid water loss by evaporation. The sections were washed with mild agitation in several baths of PBS solution, and air-dried tissue slides were dipped into liquid emulsion (Sakura NR-M2, Konishiroku Photo Ind., Co., Ltd., Tokyo), and dried at room temperature for 1 hr. To obtain rapid autoradiographic labeling the emulsion-coated slides were dipped for 10 seconds into the scintillator cocktail (7% PPO-0.02% dimethyl POPOP / diethylene dioxide), and the scintillator-impregnated emulsion was then exposed for 6~7 days at -80°C⁶⁾. The slides were developed, and stained with hematoxylin and eosin for microscopic observation. The uptake of ^3H -R5020 and its derivatives by the cells of seminiferous epithelium (SE) and interstitium (I) was measured by counting the number of reduced silver grains on the cells in the same plane focus at the top of the tissue section. The amount of radioactivity in the lumen of seminiferous tubule (L) was also measured by counting the grains over the luminal area.

RESULTS

The effect of incubation time and temperature on ^3H -R5020 binding to the tissue sections was examined. The tissue sections were incubated with 3 ng ^3H -R5020 / PBS solution at different temperatures (5 and

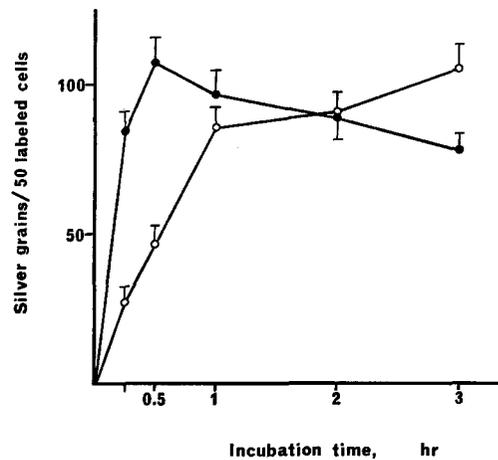


Fig. 1. Time course of ^3H -R5020 uptake by the seminiferous epithelium (SE) of the testes from 120-day-old chickens. The tissue sections were incubated with 3 ng ^3H -R5020 at 5°C (●) or at 20°C (○) (Shown are the mean \pm SE).

20°C) for different durations (0~3 hr). The incubated tissue sections were then processed to the autoradiographic analysis as described in the Methods. As shown in Fig. 1, at 5°C the number of silver grains rapidly reached a maximum by 0.5 hr, after which it decreased up to 3 hr. On the other hand, at 20°C the number of silver grains reached plateau at 2 hr, after which it remained constant up to 3 hr. The tissue sections were, therefore, incubated at 20°C for 2 hr in the present experiment.

At 2 hr after incubation of the tissue sections with ^3H -R5020, the general level of the uptake of radioactivity in the cells of SE and I was high, though differing with age (Fig. 2; Photos. 1, 3, 4, 7, 9~11). E₂B-treatment enhanced the uptake by both kinds of cells, a significant increase ($+p < 0.01$) being observed in SE in 50- and 70-day-old chickens and in I in 25-, 50- and 70-day-old chickens, (compare Photos. 7 and 9 with Photo. 10). Addition of excess dose of unlabeled R5020 significantly ($*p < 0.01$) reduced the uptake by both cell groups at each age group (Fig. 2; Photos. 2, 5, 6, 8, 12).

The uptake of radioactivity by SE and I seemed to differ with age (Fig. 2). In oil-treated animals, the uptake by SE in 50-day-old chickens was lower than that

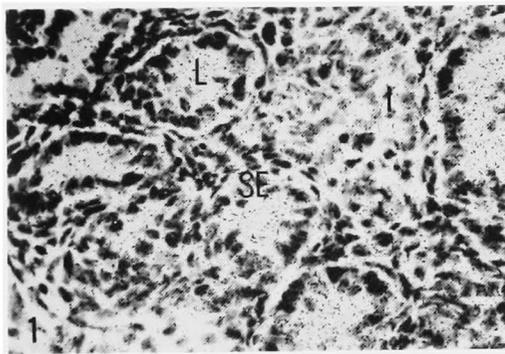
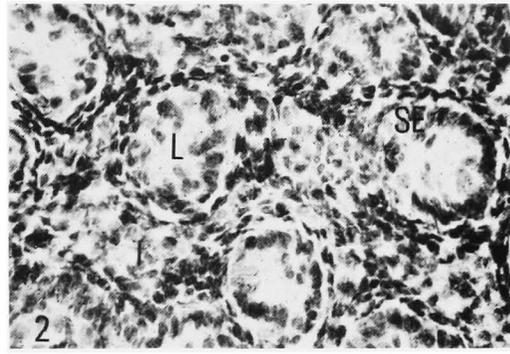
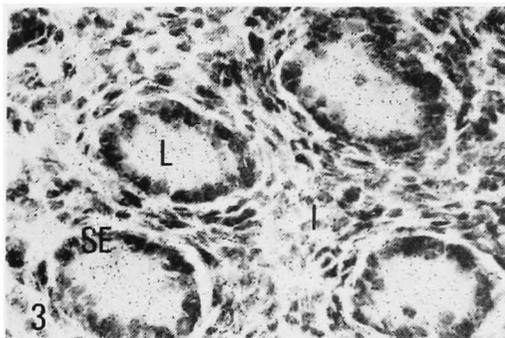
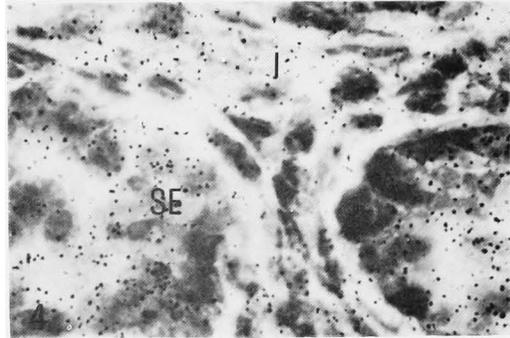
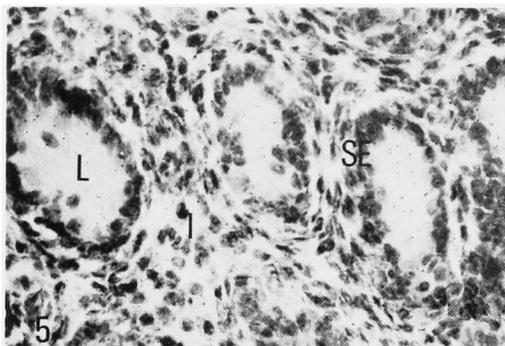
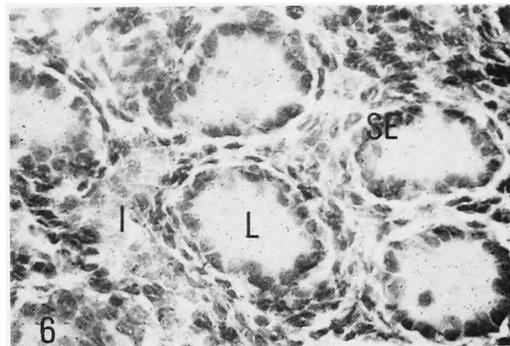
25 days. E₂B. ³H-R5020 alone25 days. E₂B. ³H-R5020+R502050 days. E₂B. ³H-R5020 alone50 days. E₂B. ³H-R5020 alone50 days. E₂B. ³H-R5020+R502050 days. oil. ³H-R5020 alone

Plate I (Photos. 1~6)

Autoradiograms of testis tissue from 25- and 50-day-old immature chickens after incubation with 3 ng ³H-R5020 in the presence or absence of 600 ng unlabeled R5020. Age of animals, treatment of animals with oil or E₂B, and incubation of the tissue sections with ³H-R5020 alone or ³H-R5020 plus R5020 are indicated under each photograph. SE=seminiferous epithelium, I=interstitium, L=lumen of seminiferous tubule. Hematoxylin-eosin stain. ×600 for Photos. 1~3, 5, 6. ×1,200 for Photo. 4 which is a magnification of Photo. 3.

at 25 days, and it was significantly (## p<0.01) higher in 70-day-old chickens, also being lower in 120-day-old chickens. The uptake by I continued to be at the same level from 25 to 70 days old, and then increased at 120 days. In E₂B-

treated animals, the uptake by SE in 50-day-old chickens (Photos. 3, 4) was lower than that in 25-day-old chickens (Photo. 1) and higher in 70-day-old chickens (Photos. 7, 9) (## p<0.01). The uptake by I showed the same fluctuation

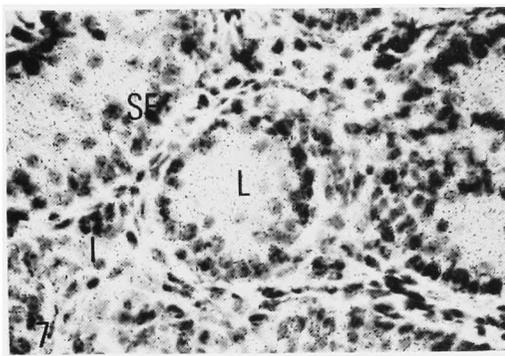
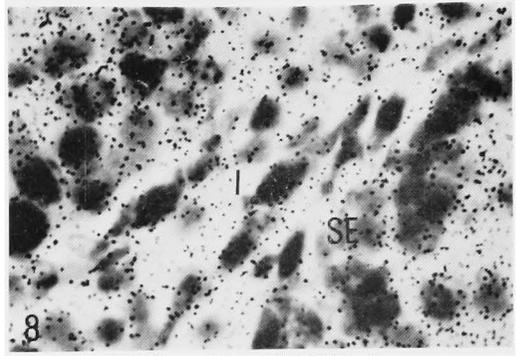
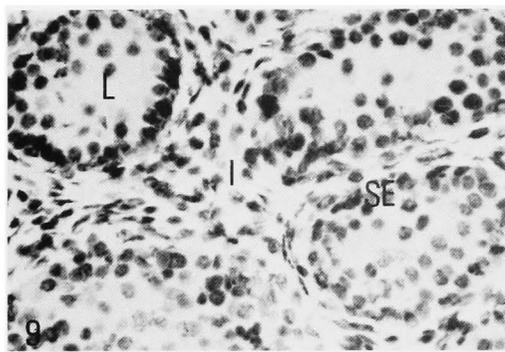
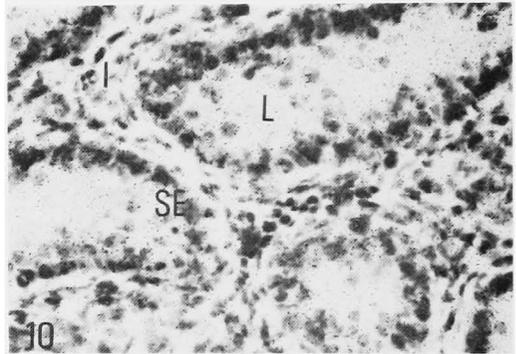
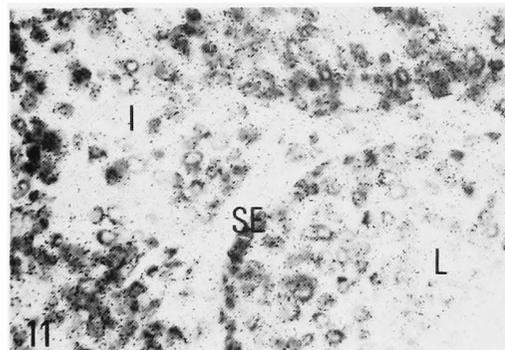
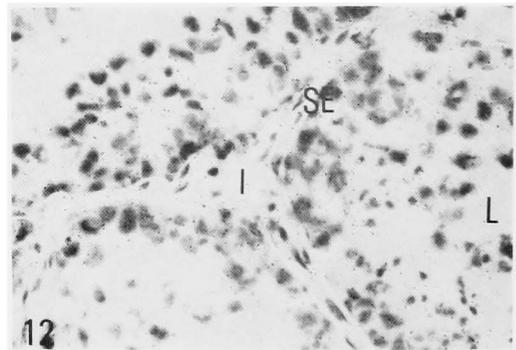
70 days. E₂B. ³H-R5020 alone70 days. E₂B. ³H-R5020 alone70 days. E₂B. ³H-R5020+R502070 days. Oil. ³H-R5020 alone120 days. Oil. ³H-R5020 alone120 days. Oil. ³H-R5020+R5020

Plate 2 (Photos. 7~12)

Autoradiograms of testis tissue from 70-day-old immature and 120-day-old mature chickens after incubation with 3 ng ³H-R5020 in the presence or absence of 600 ng unlabeled R5020. Age of animals, treatment of animals with oil or E₂B, and incubation of the tissue sections with ³H-R5020 alone or ³H-R5020 plus R5020 are indicated under each photograph. SE=seminiferous epithelium, I=interstitium, L=lumen of seminiferous tubule. Hematoxylin-eosin stain. ×600 for Photos. 7, 9~12. ×1,200 for Photo. 8 which is a magnification of Photo. 9.

as that by SE.

In both oil-and E₂B-treated chickens, the intensity of radioactivity uptake by SE tended to be higher than that by I for each age (Fig. 2; Photos. 1, 3, 4, 7, 9, 11). In E₂B-treated animals, the intensity in 70-day-

old chickens (Photos. 7, 9) was especially higher († p<0.01) in SE than in I. In oil-treated animals, the intensity in 25- and 70-day-old chickens (Photo. 10) was also especially higher († p<0.01) in SE than in I.

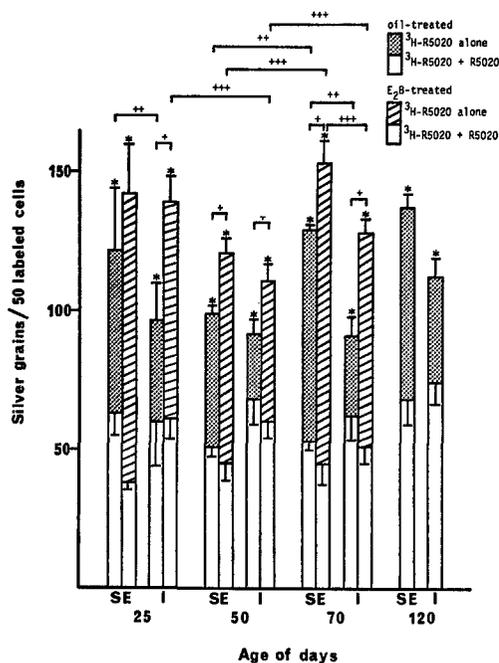


Fig. 2. Uptake of radioactivity by the seminiferous epithelium (SE) and interstitium (I) of the testis tissue from oil- and E_2B -treated chickens 2 hr after the incubation with 3 ng 3H -R5020 in the presence or absence of 600 ng unlabeled R5020. Significant differences ($P < 0.01$) between incubations with 3H -R5020 alone and 3H -R5020+R5020 are noted by (*) above top of each column. Significant differences ($P < 0.01$) in radioactivity distribution between oil- and E_2B -treated animals, between SE and I, and between age groups after incubation with 3H -R5020 alone, are noted by (+), (++) , (+++), respectively, above solid horizontal lines spanning appropriate bars (Bars over and under each column express standard error of mean).

In the SE, the intensity of the radioactivity uptake by the cells of the basal layer was stronger than that by the cells of the second and third layers (Photos. 3, 9). In the frozen sections, however, it was hard to distinguish between Sertoli cells and various spermatogenetic cells. In the I, cells with round nuclei and clear cytoplasm tended to have concentrated radioactivity (Photos. 4, 9). In the frozen sections, however, these cells could not clearly be identified as Leydig cells.

In oil- and E_2B -treated chickens, a fair

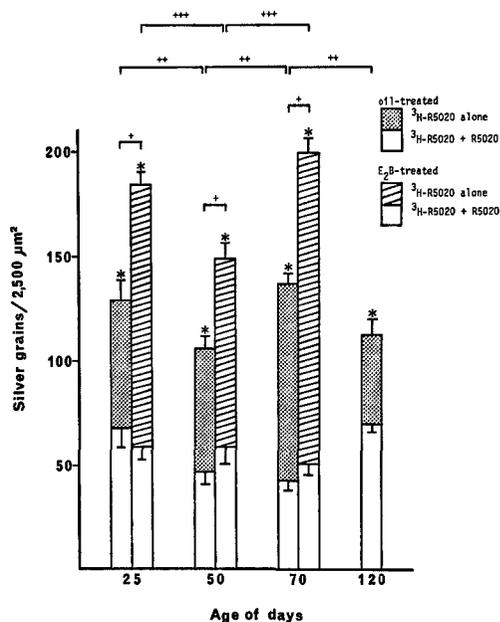


Fig. 3. Distribution of radioactivity in the lumen of seminiferous tubule (L) of the testis tissue from oil- and E_2B -treated chickens 2 hr after the incubation with 3 ng 3H -R5020 in the presence or absence of 600 ng unlabeled R5020. Significant differences ($P < 0.01$) between incubations with 3H -R5020 alone and 3H -R5020+R5020 are noted by (*) at the top of each column. Significant differences ($P < 0.01$) in radioactivity distribution between oil- and E_2B -treated animals, between age groups in oil-treated animals, and between age groups in E_2B -treated animals, are noted by (+), (++) , (+++), respectively, above solid horizontal lines spanning appropriate bars (Bars over and under each column express standard error of mean).

amount of silver grains was found in the L of the tissue sections incubated with 3H -R5020 alone (Fig. 3). The number of grains in L from the oil-treated animals differed with the age (Photos. 1, 3, 4, 7, 9, 11), less in 50-day-old chickens (++) $p < 0.01$ (Photos. 3, 4) and more in 70-day-olds (++) $p < 0.01$ (Photo. 11). E_2B -treatment significantly (+ $p < 0.01$) enhanced the radioactivity in L at each age (compare Photos. 7, 9 with Photo. 10), showing the same fluctuation pattern of radioactivity as with the oil-treatment.

Specificity of 3H -R5020 uptake by the testis tissue against various steroids was

Table 1. Steroid specificity of ^3H -R5020 uptake by the testis tissues from 120-day-old chickens†

| Unlabeled steroid added | Silver grains / 50 labeled cells (Mean \pm SE) | |
|-------------------------|--|-----------------|
| | Seminiferous epithelium | Interstitialium |
| None | 136 \pm 6 | 111 \pm 9 |
| R5020 | 68 \pm 9* | 74 \pm 7* |
| Progesterone | 76 \pm 7* | 102 \pm 13 |
| Estradio1-17 β | 128 \pm 8 | 117 \pm 7 |
| Testosterone | 123 \pm 11 | 109 \pm 6 |
| Corticosterone | 127 \pm 16 | 112 \pm 14 |

† Tissue sections were incubated for 2 hr at 20°C with 3 ng ^3H -R5020 with or without either 600 ng unlabeled steroid, and then processed to the autoradiographic analysis as described in the Methods.

* $P < 0.01$ vs none-added group.

investigated. First, the testis tissue sections from 120-day-old chickens was incubated with 3 ng ^3H -R5020 in the presence or absence of either unlabeled 600 ng R5020, progesterone, estradiol-17 β , testosterone or corticosterone. As shown in Table 1, displacement of ^3H -R5020 with R5020 and progesterone was significant ($p < 0.01$) in both SE and I, while no displacement was observed with other steroids. Next, to examine whether P-binding components in the testis tissue were contaminated with those in the blood, the blood plasma was smeared, fixed in absolute ethanol, incubated with 3 ng ^3H -R5020 / PBS solution, and then processed to the autoradiography as described in the Methods. On the smear autoradiogram, the number of silver grains was found to be reduced (Photo. not shown), indicating that the tissue P binders were not contaminated with those in the plasma.

DISCUSSION

Accumulation of radioactivity in the testis tissue sections was observed on autoradiograms as concentrated silver grains 2 hr after incubation with the radioactive R5020, a potent synthetic P. In addition, on the autoradiogram of the blood plasma smear

no visible grains were detected after incubation with ^3H -R5020. These observations clearly suggest the presence of several different types of P target cells in the chicken testes. The localization of radioactivity appears to be specific for P since addition of excess dose of unlabeled R5020 and progesterone, but not testosterone, estradiol-17 β , nor corticosterone, inhibited the accumulation of the radioactivity.

The present autoradiographic results are in agreement with the results of previous biochemical studies^{2,3} in which specific P receptors have been demonstrated in the testis cytosol of both immature 35- to 40-day-old and mature 120-day-old chickens. In the present study, the intensity of specific ^3H -R5020 uptake by SE and I fluctuated at all ages. In the SE, the fluctuation was remarkable, as compared with that in I, but the functional significance of the fluctuation by these cell constituents during the maturing periods of the chicken testes is not clear.

At 25 to 120 days after birth, E-priming enhanced the radioactivity uptake by SE and I. It is well known that the P receptor is induced by E, and thus it can serve as a marker of E action in E-sensitive tissues such as the uterus⁷, the chick oviduct⁸,

and hypothalamus and hypophysis⁹⁻¹¹). The present results may, therefore, indicate that in the chicken testis, as in other E-sensitive tissues, E acts to induce P receptors, resulting in the enhanced uptake of radioactivity by these tissues.

Stumpf¹²) earlier demonstrated nuclear concentration of ³H-estradiol in the immature rat testes. Sar et al.¹³) also demonstrated nuclear concentration of radioactivity in hypophysectomized immature rats after injection of ³H-estradiol as well as ³H-testosterone or ³H-dihydrotestosterone. They suggest that there are different receptors for estrogen and androgen in the rat testes, or that one receptor binds both estrogen and androgen. It is not known whether the P receptor in the chicken testes is different in its physicochemical nature from receptors for other steroid hormones. Judging from our results, however, this receptor is considered to bind only P because ³H-R5020 uptake was remarkably inhibited by unlabeled R5020 and progesterone, but not by testosterone, estradiol-17 β , nor corticosterone.

In the present study, the SE from 25- to 70-day-old immature chickens consisted of 1~3 cell layers of primarily Sertoli cells and spermatogonia, and that from 120-day-old mature animals consisted of 4~6 layers of these cells plus primary and secondary spermatocytes with a few spermatozoa in some tubules. Although there is an indication from the present results that Sertoli cells and spermatogonia as well as spermatocytes in the tubules accumulate R5020, further studies are needed to clearly identify P target cells. Accumulation of radioactivity by the cells with round nuclei and clear cytoplasm, probably Leydig cells, in the I also suggests a direct action of P on these cells, but, the functional significance of this hormone action on this cells remains to be clarified.

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ニワトリの精巣内プロジェスチン標的細胞の局在
オートラジオグラフによる検討

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精巣性女性化症候群患者にプロジェスチン (P)-レセプターの存在することが報告されており、この患者の精巣におけるP作用が注目される。そこで精巣に対するPの作用機序を解明する手始めに、これまでのところ系統発生の観点から各種正常動物の精巣について、*in vitro* でPレセプターの存在を検べた。その結果、ニワトリ (鳥類) の精巣でPレセプターの存在を確認したが、ウシガエル (両棲類)、トカゲ (ハ虫類) およびラット、サル、ヒト (哺乳類) では確認できなかった。

そこで本実験では、25~120日齢の成長期のニワトリの精巣におけるPレセプター含有細胞の局在とレセプター量をオートラジオグラフ手法で形態的に検討した。精巣の凍結切片標本を放射能で標識した合成P(^3H -Promegestone または ^3H -R5020) 溶液中でイ

ンキュベートした後、常法通りオートラジオグラフィをおこない、組織または細胞上の黒化銀粒子数を算定して、放射活性の強さまたはP濃度の指標とした。

その結果、1) 精細管上皮と間質細胞に放射活性の特異的集中がみられた。2) 放射活性の強さは動物の日齢によって変動した。25、70および120日齢で高く、50日齢で低かった。3) 細胞内のP濃度は動物をあらかじめエストロゲンで処置しておくで増強した。4) 放射活性の強さは間質細胞よりも精細管上皮で高かった。しかし、凍結切片標本では高放射活性を示す細胞の種類分けは困難であった。

以上のことから、成長期のニワトリ精巣の精細管壁と間質に、Pレセプター含有細胞が存在することが示唆される。レセプター含有細胞の同定は今後に残された問題である。