

Secretion of Testicular Transferrin by Cultured Sertoli Cells is Regulated by Hormones and Retinoids

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ABSTRACT

We have previously reported that Sertoli cells in culture secrete a transferrin-like protein (Skinner and Griswold, 1980). The purpose of this study was to determine to what extent hormones such as follicle-stimulating hormone (FSH), insulin and testosterone, and vitamin A can regulate the amount of testicular transferrin secreted by cultured Sertoli cells.

Sertoli cell culture medium was collected every 48 h for the duration of culture and the amount of testicular transferrin in the medium was quantitated with a radioimmunoassay. The maximum amount of transferrin was detected in cell cultures which had been maintained in medium supplemented with FSH, insulin, retinol, and testosterone. During the first 48 h of culture these cells secreted 65 ± 10 ng transferrin/ 10^5 cells compared to control cultures which secreted 56 ± 8 ng transferrin/ 10^5 cells. In the 48 h collection ending on Day 6 of culture, the amount of transferrin secreted into the medium had risen to 164 ± 16 ng transferrin/ 10^5 cells in treated cultures compared to 25 ± 5 ng transferrin/ 10^5 cells in control cultures. It was found that the secretion of transferrin was regulated by the interaction of insulin with FSH, testosterone and retinol. When insulin was present in the medium, the cells, if untreated or treated with FSH or testosterone, secreted nearly twice as much transferrin as when the insulin was deleted. Both retinol and retinoic acid stimulated the secretion of transferrin by cultures maintained in medium which contained insulin.

The secreted proteins were labeled with [³⁵S] methionine and analyzed by two-dimensional gel electrophoresis and fluorography. Untreated Sertoli cell cultures secreted nearly undetectable amounts of [³⁵S] methionine-labeled transferrin while cultures treated with FSH, insulin, testosterone and retinol secreted a greatly increased amount of radioactive transferrin.

Transferrin secretion was also stimulated when calf serum was added to the medium. It was found that as the concentration of serum was increased, the relative response of the cells to FSH, insulin, testosterone, and retinol decreased. As the percentage of calf serum was increased to 10%, the amount of secreted transferrin in the medium approached a constant level which was equivalent to that amount of transferrin secreted by serum-free cultures which were maintained in FSH, insulin, testosterone and retinol. Sertoli cell cultures from 10, 20 and 60-day-old rats all secreted transferrin. No detectable transferrin was secreted by cultured peritubular fibroblasts.

These results demonstrate that the secretion of transferrin by Sertoli cells in culture is regulated by a complex interaction of hormones, vitamin A and serum factors.

INTRODUCTION

The Sertoli cells of the mammalian testis are secretory cells that play a major role in the maintenance and control of spermatogenesis (Fawcett, 1975; Fritz, 1973). Tight junctional complexes between Sertoli cells create a "blood-testis barrier," thus providing a unique environment in the lumen of the seminiferous tubule (Fawcett, 1975; Waites, 1977). Sertoli cells secrete glycoproteins into the lumen of the tubule and perhaps into the blood stream or the lymph, and a number of important biological

functions have been attributed to these secreted glycoproteins (Waites, 1977; Hagenas et al., 1975; Gunsalus, 1978). The synthesis and secretion of the glycoproteins have been studied utilizing cultured Sertoli cells (Wilson and Griswold, 1979) and three of the secreted proteins from rat Sertoli cell cultures have been identified as androgen binding protein (ABP) (Fritz, 1976), plasminogen activator (Lacroix et al., 1977), and testicular transferrin (tTf) (Skinner and Griswold, 1980). Of these proteins only ABP has been purified and characterized (Feldman et al., 1981).

The detectable activity of both plasminogen activator and ABP was greatest in cultures which were supplemented with FSH (Fritz et al., 1976; Louis and Fritz, 1979) and the

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optimal serum-free conditions for the maintenance of ABP secretion by Sertoli cells consisted of medium which was supplemented with FSH, insulin, testosterone, and retinol (Karl and Griswold, 1980a; Karl and Griswold, 1980b).

Testicular transferrin appears to be a major Sertoli cell product and the synthesis and secretion of transferrin may have great importance in spermatogenesis (Skinner and Griswold, 1980). We have established a radioimmunoassay for the transferrin which is secreted by Sertoli cells in culture and we have utilized this radioimmunoassay to monitor the response of Sertoli cells to hormones and retinoids. The results have enabled us to clarify the role of some of these agents in Sertoli cell functions.

MATERIALS AND METHODS

Chemicals

Medium for cell culture (Ham's F-12) was made from powdered formulation supplied by GIBCO. Calf serum was also supplied by GIBCO. Purified rat transferrin and rabbit-anti-rat transferrin were obtained from Cappel Labs. ^{125}I and ^{35}S methionine were obtained from New England Nuclear Corp. FSH (NIAMDD ovine FSH-S13) was obtained from the National Pituitary Agency, National Institute of Health. All other chemicals were obtained from Sigma Chemical Co.

Cell Culture

Sertoli cells from 20-day-old rats (unless otherwise specified) were prepared and cultured essentially as previously described (Wilson and Griswold, 1979). Cells were cultured in F-12 medium in 24-well Linbro dishes (Linbro Scientific, Inc.). Approximately 5×10^5 cells were plated per well and the cells were maintained in the absence of calf serum unless otherwise specified. The medium (1 ml) from each well was collected and replenished every 2 days for the duration of culture. Collected samples of medium were stored at -20°C until assayed. Hormones and vitamin A were added to designated cultures in the following final concentrations: insulin (5 $\mu\text{g}/\text{ml}$), FSH (25 ng/ml), testosterone (0.7 μM), and retinol (0.35 μM). The number of cells in each culture was determined in a Coulter ZF cell counter after the cells were removed from wells with 0.5 ml of 1.7% trypsin solution in isotonic saline.

Peritubular cell cultures were also prepared as previously described and were subcultured in F-12 medium which contained 10% calf serum (Wilson and Griswold, 1979).

Iodination

Rat serum transferrin was iodinated with ^{125}I by the use of chloramine T as described by Greenwood et al. (1963). A 0.5 M phosphate buffer, pH 7.5, was utilized in all the iodination solutions. Transferrin (5

μg in 10 μl buffer) was added to 20 μl of phosphate buffer containing 2 mCi of ^{125}I . The reaction mixture was vortexed 120 sec following the addition of 10 μl of 1.5 mg/ml chloramine T solution. Upon completion of the reaction 50 μl (7.8 mg/ml) sodium-metabisulfite was added. The solution was then transferred to a 1 X 10 cm BioRad P-6 column with 100 μl of 16% sucrose in 10% KI. The column was eluted with a gelatin buffer (50 mM Tris, pH 7.5; 2.5 mg/ml Sigma Type I, 300 bloom swine gelatin buffer 0.15 M NaCl, 10 mM EDTA). Approximately 5 $\mu\text{Ci}/\mu\text{g}$ of iodinated transferrin was obtained.

Radioimmunoassay

A quantitative assay of testicular transferrin was developed using a rabbit anti-rat serum transferrin. The gelatin buffer (see above) was used in all solutions unless otherwise specified and assays were done in 12 X 75 mm glass disposable culture tubes. Standards were made from a rat serum transferrin solution stored at -70°C . Diluted standards were stored up to 2 weeks at -20°C and contained 10 to 150 ng transferrin in 100 μl solution. In all unknowns and standards the total amount of medium (F-12) assayed was 0.5 ml.

Rat transferrin antibody (100 μl of a fresh 1/100 dilution of a 1 mg/ml solution of IgG) was added to the unknowns and standards. After the addition of 100 μl iodinated transferrin containing 30,000 cpm, the volume in each tube was 1.55 ml. Following an incubation for 1 h at 37°C , 100 μl of a 1/30 dilution of goat anti-rabbit serum and 100 μl of a 1/400 dilution of normal rabbit serum were added (final volume 2.25 ml per tube). After another 1 h incubation at 37°C , 1 ml polyethylene glycol buffer (132 mg/ml polyethylene glycol 4000, 50 mM Tris pH 7.5) was added (Hao and Wickerhauser, 1978; Wood, 1980). After an incubation at room temperature for 15 min, the samples were centrifuged at $3000 \times g$ for 2 h at 4°C . The radioactivity in the pellet was determined in a Beckman Gamma 4000 counter with the aid of a Beckman DP 5000 data reduction system.

Gel Electrophoresis

Sertoli cell cultures were labeled with ^{35}S methionine (10 $\mu\text{Ci}/\text{ml}$, 500 Ci/mMole) in F-12 medium which lacked methionine. The cells were incubated with the isotope for 12 h on Day 5 of culture. The medium was collected on Day 6 and was concentrated and desalted as previously described (Wilson and Griswold, 1979). The secreted proteins were then separated by two-dimensional gel electrophoresis according to O'Farrell (1975). After completion of the second dimension (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) the gels were dried and fluorographed (Wilson and Griswold, 1979; Bonner and Laskey, 1974; Laskey and Mills, 1975). Approximately 2×10^5 cpm of ^{35}S methionine-labeled proteins were initially placed on the gel and the fluorography was generally completed within 48 h.

RESULTS

Characteristics of the Radioimmunoassay

The antibody used in the radioimmunoassay was directed against rat serum transferrin. Rat

testicular transferrin and rat serum transferrin have a similar molecular weight (Skinner and Griswold, 1980) and similar isoelectric points (Kissinger, 1981). The displacement of iodinated transferrin from anti-transferrin antibodies by rat serum transferrin and by rat testicular transferrin is shown in Fig. 1. The parallel displacement curves imply that the transferrins from the serum and from the testes are antigenically similar (Midgeley et al., 1968). The sensitivity of the radioimmunoassay was determined to be approximately 10 ng transferrin. Nonspecific binding was approximately 2% of the added radioactivity and the intra- and inter-assay coefficients of variations were 6% and 10%, respectively. Culture medium (10-fold concentrated), 1% to 10% calf serum, and all the hormones and vitamins utilized in the cell culture were assayed and found to have no detectable cross-reactivity in the transferrin radioimmunoassay. It has been demonstrated previously that only one protein (testicular transferrin) secreted by Sertoli cells can be immunoprecipitated with rat serum transferrin antibody (Skinner and Griswold, 1980).

Regulation of Transferrin Secretion

We have previously reported that a combination of hormones (FSH, testosterone and insulin) and a vitamin (retinol) would maintain the production of androgen binding protein (ABP) in cultured Sertoli cells (Karl and Griswold, 1980b). We now report the results of investigations on the action of these agents on transferrin secretion. Sertoli cells were prepared from 20-day-old rats and cultured in the presence or absence of hormones and vitamin A. The number of cells per culture plate was found to be independent of hormone additions but declined linearly by 17–20% from Day 2 to Day 8 (data not shown). The cells were counted on Day 8 of culture and the data for the amount of transferrin secreted per 10^5 cells has been corrected to reflect the actual number of cells on the plate at the time of collection. The results of the radioimmunoassay for transferrin in the collected medium is shown in Fig. 2. The secretion of transferrin by the cell cultures was initially stimulated by the addition of FSH, insulin, retinol, and testosterone (FIRT) to the medium. After 6 days in culture the appearance of transferrin in the medium was reduced. The delayed addition of hormones and retinol at Day 4 of culture also stimulated the secretion

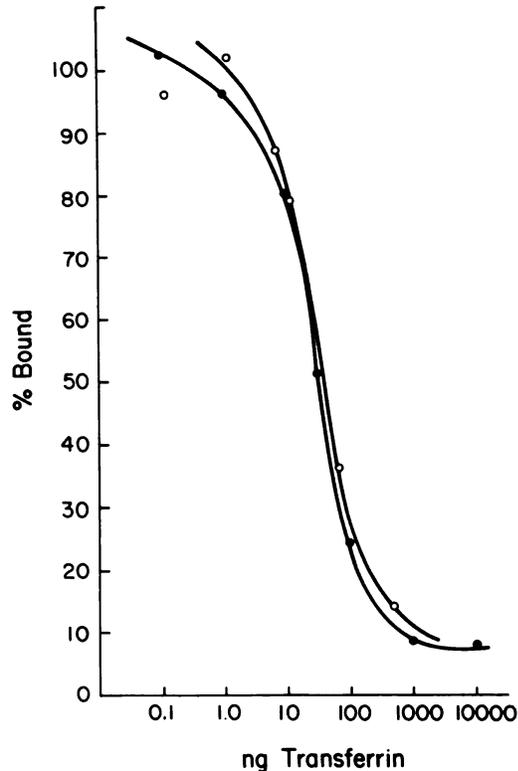


FIG. 1. Displacement of the binding of ^{125}I rat serum transferrin to an antibody to rat serum transferrin. The radioimmunoassay method described previously was used for both assays. The bound ^{125}I rat serum transferrin was displaced by increasing amounts of unlabeled rat serum transferrin (●-●) or testicular transferrin (○-○). Testicular transferrin was diluted from a stock of 100-fold concentrated Sertoli cell culture medium. Data is expressed as the percentage of ^{125}I transferrin which is immunoprecipitated in the radioimmunoassay. Each point is the mean of 6 determinations which had a coefficient of variation of 15% or less.

of transferrin into the medium but the magnitude of the stimulation was smaller.

Also shown in Fig. 2 are the results of assays of transferrin in cultured cells which were scraped from the culture plates on Day 6 of culture, washed, and homogenized in 10 mM Tris pH 7.5. There was no detectable transferrin in the cell homogenates from untreated cultures while the homogenates from FIRT-treated cells contained only 15 ng transferrin/ 10^5 cells. Culture medium (100-fold concentrated) from subcultured peritubular cells was devoid of detectable transferrin (data not shown).

The contribution of an individual hormone

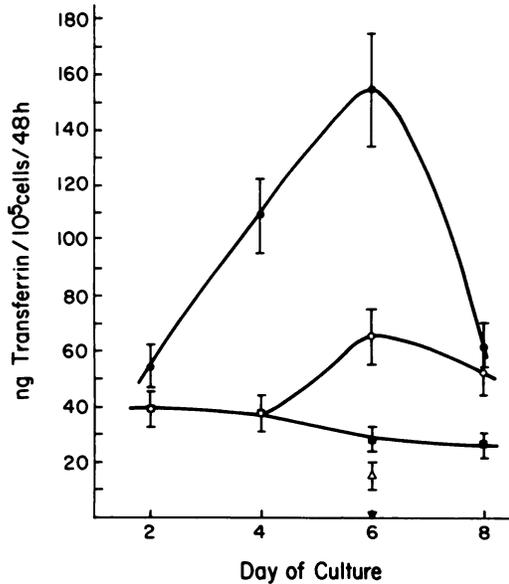


FIG. 2. Stimulation of the secretion of testicular transferrin from cultured Sertoli cells. Medium was changed and collected every 48 h from Sertoli cells cultured from 20-day-old rats. The medium was assayed for testicular transferrin by a radioimmunoassay. The cultures were maintained in medium alone (■-■), in medium supplemented with FSH, insulin, testosterone and retinol (●-●) or in medium alone for 4 days with the subsequent addition of FSH, insulin, testosterone and retinol after Day 4 of culture (○-○). Each point represents the mean and standard deviation of 8 separate determinations. After 6 days some cultures were terminated; the medium was removed, and the cells were homogenized. The amount of transferrin in the cells is shown for control cultures (▼) and for FIRT-treated cultures (Δ). The number of cells per culture was determined on Day 8 and the data for preceding culture periods was adjusted for loss of cells as described in the text.

or vitamin to the stimulation of transferrin secretion was examined by the radioimmunoassay (Fig. 3). The results of this experiment suggested that one hormone, insulin, influenced the ability of the cell to respond to each of the other hormones and retinol. Figure 3 shows the secretion of transferrin by Sertoli cell cultures after the addition of only one agent (panel A) or combinations of these agents (panel B). FSH alone stimulated transferrin secretion, but neither testosterone nor retinol had much effect. In panels C and D the results are shown for the synthesis of transferrin in the presence of insulin (5 $\mu\text{g/ml}$) in addition to the same agents or combinations of agents. In the presence of insulin both retinol and testosterone

appear to stimulate transferrin secretion.

When the magnitude of the response of Sertoli cell cultures was analyzed it was apparent the relative response of the hormone-treated cultures to the control cultures was nearly unchanged but that insulin stimulated the response of Sertoli cells to F, T, FT, or FTR by nearly a constant factor of $200\% \pm 30\%$ (Table 1). Retinol appeared to be an exception to this observation because the retinol-treated cultures were stimulated more than 300% if maintained in the presence of insulin (Tables 1 and 2). Retinoic acid stimulates the secretion of transferrin even more effectively than retinol (Table 2).

The concentration of each individual hormone and retinoid required to elicit a maximum secretion of transferrin from the Sertoli cells was determined. An investigation of the retinoids showed that both retinol and retinoic acid have similar dose-response relationships and give a half-maximal response at approximately $0.10 \mu\text{M}$ (Fig. 4).

Testosterone has a small but reproducible stimulatory effect on transferrin synthesis in the presence of insulin or insulin and FSH. In some experiments the stimulatory effect of testosterone was more pronounced but seldom exceeded 130% of the control values. The concentration for half-maximal responses of the Sertoli cells to insulin was found to be approximately $0.2 \mu\text{M}$ (Fig. 5). It was difficult to estimate the half-maximal concentration of testosterone which was required but the value appeared to be between 0.02 and $0.12 \mu\text{M}$ (Fig. 5).

Some of the dose-response characteristics of FSH stimulation of transferrin synthesis were also examined (Table 3). At very low concentrations of FSH (less than 25 ng/ml), the response of the Sertoli cells varied from preparation to preparation of cells so that an exact dose-response curve could not be obtained. However, at concentrations of FSH of 25 ng/ml or greater (up to $1 \mu\text{g/ml}$), the response of the Sertoli cells was constant (Table 3). The dose-response relationships for FSH, retinol, retinoic acid and testosterone were obtained from cultures which were maintained in $5 \mu\text{g/ml}$ of insulin. The deletion of insulin from the medium did not alter the dose-response curves but lowered the maximum response of the cells (data not shown).

The secreted proteins from control and FIRT-treated Sertoli cell cultures were labeled

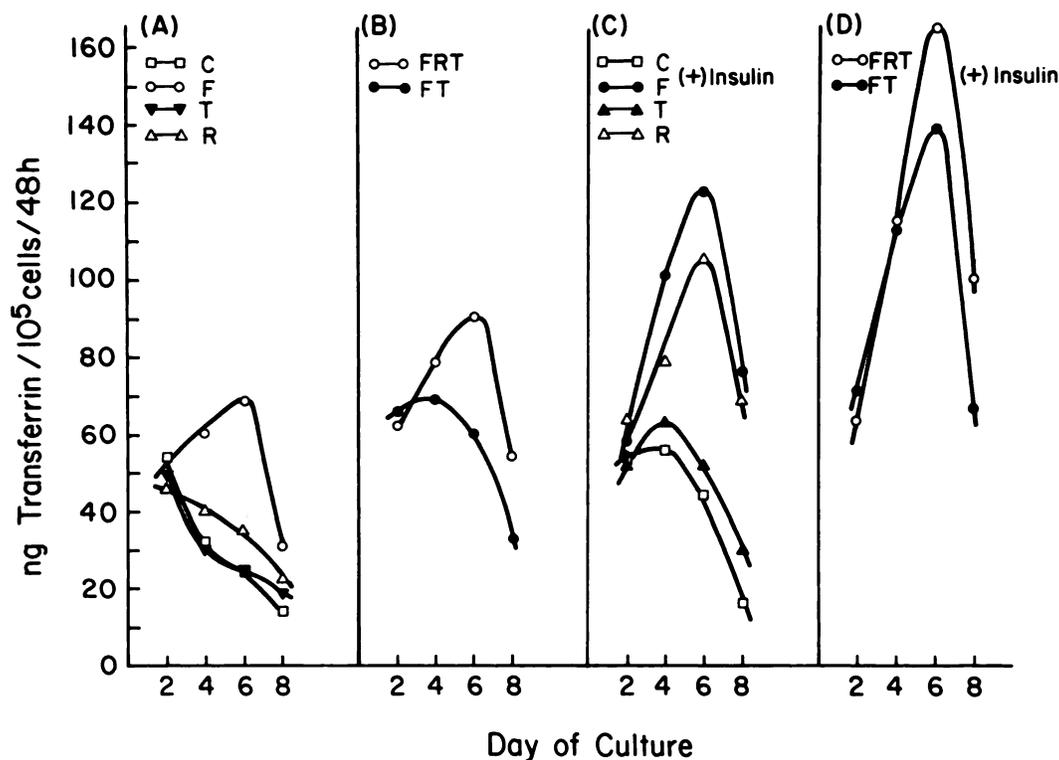


FIG. 3. Regulation of transferrin secretion from Sertoli cells cultured in the presence of different combinations of hormones. Sertoli cells from 20-day-old rats were cultured as described. The medium was collected every 48 h and the amount of testicular transferrin was determined by a radioimmunoassay. The hormones and vitamin A were added to the medium at the time of cell plating and re-added each time the medium was collected and replaced. Each point represents the mean of values obtained from 12 culture wells. These 12 culture wells were from triplicate cultures from 4 separate preparations of cells. The coefficient of variation of each point is less than 15%. In C and D, insulin (5 $\mu\text{g}/\text{ml}$) was added to the F-12 culture medium. In addition to insulin, FSH (F), testosterone (T), or retinol (R) or combinations (FT, FRT) of these agents were added to designated cultures. Untreated cultures are designated as control (C).

TABLE 1. Relative hormonal stimulation of transferrin secretion as a function of the absence or presence of insulin. Data was taken from the values shown for Day 6 of culture in Fig. 3.

Treatment	- Insulin		+ Insulin		Ratio (+) insulin (-) insulin
	ng Transferrin 10^5 cells	Ratio treated/ control	ng Transferrin 10^5 cells	Ratio treated/ control	
FSH (F)	69 ± 10^f *	2.8	122 ± 18^c	2.7	1.8
Retinol (R)	35 ± 5^i	1.4	105 ± 15^d	2.3	3.0
Testosterone (T)	26 ± 5^{ij}	1.0	52 ± 8^{gh}	1.2	2.0
F, T	60 ± 9^{fg}	2.4	138 ± 19^b	3.1	2.3
F, R, T	90 ± 14^e	3.6	164 ± 22^a	3.6	1.8
None	25 ± 5^j	...	45 ± 7^h	...	1.8

*The values in each column and the values in each major group (\pm insulin) were analyzed statistically with Duncan's multiple range test for variable values. The values with different superscript letters are significantly different from each other at $P < 0.05$ (Duncan, 1955).

TABLE 2. Stimulation of testicular transferrin secretion by retinol and retinoic acid in the presence and absence of insulin. Sertoli cells were prepared from 20-day-old rats and cultured as described.

Treatment	Concentration	ng Transferrin/10 ⁵ cells/48 h
Control	...	25 ± 5 ^{f*}
Insulin	5 µg/ml	45 ± 7 ^d
Retinol	0.35 µM	35 ± 5 ^e
Retinol, insulin	Same as above	105 ± 16 ^b
Retinoic acid	0.33 µM	55 ± 8 ^c
Retinoic acid, insulin	Same as above	146 ± 21 ^a

*The data shown are the mean and standard deviations from 9 determinations which were done on medium from the 48-h culture period which encompassed Day 5 and 6 of culture. The number of cells per culture was determined on Day 6 of culture. The values were evaluated statistically with Duncan's test and values with different superscript letters are significantly different from each other at P<0.05 (Duncan, 1955).

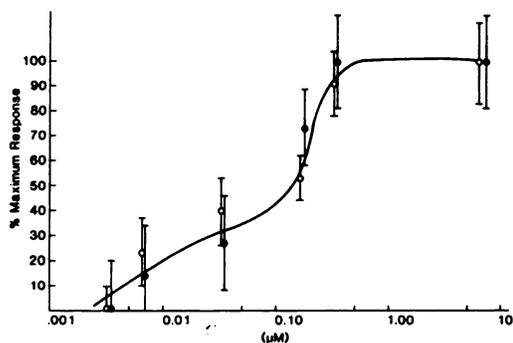


FIG. 4. Dose-response curves for the stimulation of transferrin secretion by retinol and retinoic acid (A). Sertoli cells were cultured as described in the text and the amount of transferrin in the medium was determined on a sample collected from Days 4-6 in culture. Each point represents the mean value and standard deviation from 6 separate determinations on retinol (●—●) or retinoic acid (○—○) treated cultures.

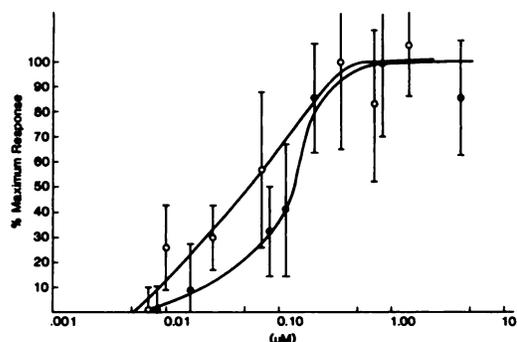


FIG. 5. Dose-response curves for the stimulation of transferrin secretion by testosterone and insulin. The experiment was the same as described in the legend of Fig. 4 except that the cultures were treated with insulin (●—●) or testosterone (○—○).

with [³⁵S]methionine and analyzed by two-dimensional electrophoresis (Fig. 6). The location of testicular transferrin on the gel was determined by an analysis of immunoprecipitated testicular transferrin by a similar procedure (Skinner and Griswold, 1980). Serum transferrin appears at the same coordinates on a two-dimensional gel as does testicular transferrin (Kissinger, 1981). It is clear from the fluorograms that FIRT treatment of the cells significantly increases the amount of [³⁵S]methionine incorporated into secreted transferrin. At the time during culture when the cells were

TABLE 3. The secretion of transferrin by sertoli cells cultured in the presence of different concentrations of FSH.

Con Concentration of FSH	ng Transferrin/10 ⁵ cells
0 (12)	70 ± 11 ^{c*}
.05 ng/ml (9)	75 ± 11 ^c
2.5 ng/ml (9)	102 ± 10 ^b
25 ng/ml (12)	132 ± 18 ^a
250 ng/ml (3)	150 ± 21 ^a
1 µg/ml (6)	125 ± 14 ^a

*Each value represents the mean and standard deviation with the number of determinations shown in parentheses. Cells were maintained in 5 µg/ml insulin and transferrin determinations were done on medium from the 48-h period which included Days 5 and 6 of culture. The values were evaluated with Duncan's test and values with different superscript letters are significantly different from each other at P<0.05 (Duncan, 1955).



FIG. 6. Two-dimensional gel electrophoresis of [35 S] methionine-labeled secreted proteins. Sertoli cell cultures were prepared from 20-day-old rats. Some of the cultures were maintained in medium (control) and some in medium which contained FSH, insulin, testosterone and retinol. On the fifth day of culture the F-12 medium was replaced with medium supplemented with [35 S] methionine. After 12 h of incubation the medium was collected, and the secreted proteins were concentrated and desalted. The secreted proteins were then analyzed by two-dimensional electrophoresis according to O'Farrell (1975). In the first dimension the pH gradient was from 3.5 to 8.0 and in the second dimension the acrylamide gradient was from 5% to 15%. The electropherograms were then fluorographed for 24 to 48 h. Analysis of secreted proteins from control cultures (A) and from FIRT-treated cultures (B) are shown. The *arrow* denotes the location of testicular transferrin.

labeled (Day 5–6), almost no detectable incorporation of [³⁵S]methionine into transferrin could be observed in control cultures (Fig. 6).

The secretion of transferrin by cultured Sertoli cells from 20-day-old rats was also stimulated by the addition of small amounts of calf serum to the culture medium. As little as 0.05% calf serum in the medium significantly stimulated transferrin secretion. As the percentage of calf serum in the culture medium was progressively increased, the secretion of transferrin approached a constant level which was equivalent to that amount of transferrin secreted by FIRT-treated cultures in serum-free medium (Fig. 7).

Sertoli cells were prepared from rats of 10, 20 and 60 days of age. The transferrin secreted by these cultures in serum-free medium was assayed on the second day of culture because the cultures from 10-day-old rats are short-lived in the absence of serum or hormones. The cells from all age groups synthesized and secreted transferrin into the medium (Table 4). However, the secretion of transferrin in the cultures from 10-day-old or 20-day-old, but not 60-day-old, rats was stimulated by the addition of FIRT. The amount of transferrin secreted by the Sertoli cells appeared to increase as the age of the donor rat increased. If these cultures were maintained with 1% serum in the medium, they were viable for at least 6 days of culture (Table 4).

DISCUSSION

Sertoli cells from 10- and 20-day-old rats responded in culture to FSH, insulin, testos-

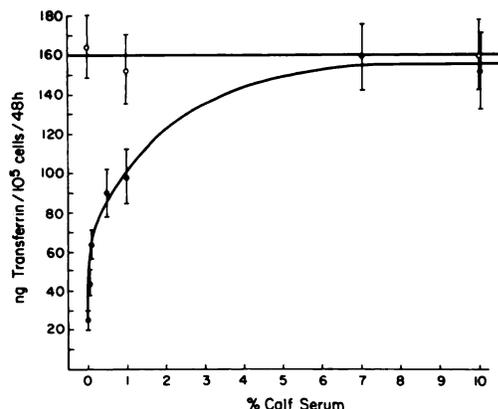


FIG. 7. Stimulation of testicular transferrin secretion by increasing amounts of calf serum. Sertoli cells from 20-day-old animals were cultured as described. In addition, various amounts of calf serum were added to the medium at the day of plating. The medium, hormone, vitamin, and sera additions were all replaced every 48 h. The data shown are the amounts of transferrin secreted by the cultures during the 48 h period encompassing Days 5 and 6 of culture. The number of cells per culture was determined after Day 6 of culture. Each point represents the mean value and standard deviation from 6 separate determinations on control cultures (●—●) and FIRT-treated cultures (○—○).

terone and retinol with an increased secretion of testicular transferrin. The maximum amount of transferrin secreted by Sertoli cells under these conditions was approximately 160 ng transferrin per 10⁵ cells per 48 h. This amount corresponds to approximately 2 pmoles of transferrin/10⁵ cells/48 h or about 100 fmoles of transferrin per μ g of cell DNA per h (assuming

TABLE 4. Transferrin secretion by sertoli cells cultured from rats of different ages. Cultures and transferrin radioimmunoassays were as described.

Treatment	Day of medium collection	Serum concentration	Age of rat (days) (ng Tf/10 ⁵ cell/48 h)		
			10	20	60
Control	2	0	20 ± 5 (6) ^{h*}	55 ± 5 (12) ^f	130 ± 25 (6) ^c
FIRT	2	0	43 ± 9 (6) ^g	105 ± 16 (12) ^d	135 ± 26 (6) ^c
Control	6	1%	96 ± 16 (9) ^d	97 ± 14 (12) ^d	62 ± 12 (9) ^e
FIRT	6	1%	180 ± 30 (9) ^a	151 ± 23 (14) ^b	64 ± 13 (9) ^e

*Each value represents the mean and standard deviation with the number of separate determinations shown in parentheses. Cells were maintained in the presence or absence of hormones and vitamin A and in the presence of 1% calf serum. The number of cells per culture was determined on the day the medium was collected. All values were compared statistically with Duncan's test and values with different superscript letters are significantly different at $P < 0.05$ (Duncan, 1955).

4 pg DNA/cell). In contrast, the maximum amount of ABP secreted by Sertoli cells cultured in 24-well plates has been reported to be approximately 1.6 fmoles ABP per μg of cell DNA per h (Louis and Fritz, 1979). When cells were cultured in 25 cm^2 dishes in the presence of FIRT, the maximum amount of ABP secreted was only 0.5 to 1.0 fmoles per μg of cell/per DNA h (Karl and Griswold, 1980). It has also been shown that the rate of secretion of ABP by cultured Sertoli cells continually decreases with time in culture (Karl and Griswold, 1980b; Rommerts et al., 1978). The addition of hormones such as FSH, testosterone and insulin to cultured Sertoli cells resulted only in a slower decrease in the preexisting rate of ABP secretion. In contrast to ABP, the amount of transferrin secreted by these cells was markedly stimulated by these hormones.

The increased accumulation of transferrin in the culture medium could be due to an increased synthesis of transferrin, an increased rate of glycosylation and secretion of transferrin or a combination of these factors. It cannot be attributed to the increased release of preexisting transferrin molecules because there is very little immunoreactive transferrin sequestered inside the cells (Fig. 2). The analysis of the secreted proteins by two-dimensional gel electrophoresis and fluorographs suggests that the hormones and vitamin A stimulated the net synthesis of transferrin. The treatment of cells with FIRT results in a greatly increased amount of [^{35}S]methionine incorporation into testicular transferrin. It also appears that the effect of the hormone treatment of the cells is quite specific. The major difference between two-dimensional electrophoresis of control and treated cells was the amount of isotope which appeared in the spot corresponding to transferrin.

The exact half-maximal concentration of FSH required to stimulate the transferrin secretion was not determined. Clearly, 25 ng/ml of FSH was sufficient to maximally stimulate transferrin secretion. It is likely that the concentration of FSH required for one-half maximum stimulation of transferrin secretion is similar to the value reported by Fritz et al. (1978) for ABP secretion (4.02 ng/ml NIH-FSH-S11). However, it has been shown that different biochemical functions of the Sertoli cells require different concentrations of FSH to elicit a half-maximal response (Fritz et al., 1978). The concentration of testosterone required to stimulate transferrin synthesis to a

half-maximal value appeared to be somewhat higher than the 4 nM value which has been reported to be required for half-maximal stimulation of ABP synthesis in cultured Sertoli cells (Louis and Fritz, 1979).

The action of insulin on cultured Sertoli cells seemed to be independent of and additive with the combined actions of FSH, testosterone and the retinoids. The presence of insulin in the medium increased the basal level of transferrin secretion approximately 2-fold. In cultures which were maintained in insulin, the relative stimulation of transferrin secretion, by FSH and testosterone or combinations of FSH, testosterone and retinoids, over the basal level of secretion was correspondingly increased. Thus the percentage stimulation of transferrin secretion by FSH or testosterone in these cultures was the same as in cultures which did not contain insulin. The biochemical mechanism by which insulin potentiates the response of the Sertoli cells is unknown. It was necessary to use micromolar concentrations of insulin in our cultures and the growth of many cell types in culture has been shown to require a similar nonphysiological concentration of this peptide (Hayashi et al., 1978; Hayashi and Sato, 1976). It has also been shown that insulin can rapidly lose activity in the usual serum-free tissue culture medium (Hayashi and Sato, 1976). Thus, it is not clear whether the action of insulin on Sertoli cells is a direct result of the interaction of insulin molecules with insulin receptors or is the result of an indirect effect due to the relatively high concentration of insulin in the culture medium. It is possible that insulin may mimic the biological activity of a related peptide such as nonsuppressible insulin-like activity or sommatomedin (Barnes and Sato, 1980).

The action of retinol on transferrin synthesis is consistent with the known requirement of retinol for the maintenance of normal spermatogenesis (Thomson et al., 1964). The importance of retinol in glycoprotein synthesis has been well documented (for a review see Lotan, 1980). In addition, Sertoli cells contain very high levels of cytoplasmic retinol binding protein (Huggenvik and Griswold, 1981). The stimulation of transferrin synthesis by retinoic acid was unexpected because the acid form of the retinoids is ineffective in maintaining spermatogenesis in vitamin A deficient rats (Thomson et al., 1964). Appling and Chytil (1981) have recently presented evidence which

suggests that retinoic acid does have a role in interstitial functions. Our data would suggest that both retinol and retinoic acid can act on cultured Sertoli cells. Even though retinoic acid can stimulate transferrin secretion more than retinol can, the effective concentrations of these two retinoids are similar. The concentration of retinoids required to elicit a half-maximal response from Sertoli cells is consistent with that shown for other vitamin A sensitive cells (Lotan, 1980).

After 6 days of culture the amount of transferrin synthesized by the hormone-treated cells declined. The reason for the decreased synthesis is not known but was probably related to the cell culture conditions.

Calf serum stimulated the secretion of transferrin at concentrations as low as 0.05%. The secretion increased to a maximum at approximately 7 to 10% calf serum. As the concentration of calf serum was increased the magnitude of the response of Sertoli cell cultures to FSH, insulin, testosterone and retinol declined. In 7–10% calf serum essentially no hormone stimulation of transferrin secretion was observed. The Sertoli cell cultures reached a maximum rate of transferrin secretion (approximately 160 ng Tf per 10^5 cells per 48 h on Day 6 of culture) that could not be surpassed. The effect of calf serum on the secretion of transferrin is most likely due to a combined effect of growth factors, hormones, and vitamins present in calf serum. The relatively decreased hormone responses in the presence of calf serum suggest that precaution must be used when hormone regulation in cell culture is studied in the presence of serum.

Seminiferous tubules from 10-day-old rats have no microscopically discernable lumen into which glycoproteins can be secreted and Sertoli cell preparations from these rats produced a relatively low amount of transferrin. After a few days of culture in the presence of hormones and 1% calf serum, the Sertoli cells from 10-day-old rats secrete transferrin at a rate which was more nearly equivalent to that of Sertoli cells from 20-day-old animals. Sertoli cells from 60-day-old rats secreted transferrin but did not respond to FSH, insulin, testosterone or retinol. It has been reported that Sertoli cells from 60-day-old rats do not respond to FSH, presumably because they contain high levels of phosphodiesterase activity (Means, 1977). It is also possible that Sertoli cells from 60-day-old rats are already maximally stimulated

to synthesize transferrin before hormones are added. After 2 days in culture the secretion rate of Sertoli cells from 60-day-old rats is decreased because these cells do not survive as well in culture as cells from younger animals.

The importance of transferrin secretion by Sertoli cells to spermatogenesis remains to be fully elucidated. However, it appears that the secretion of transferrin can be utilized as a convenient and sensitive indication of Sertoli cell responses in cell culture.

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