BIOLOGY OF REPRODUCTION 27, 233–240 (1982)

Analysis of Sertoli Cell-Secreted Proteins by Two-Dimensional Gel Electrophoresis

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ABSTRACT

The [^{35}S] methionine-labeled proteins secreted from cultured Sertoli cells were analyzed by two-dimensional gel electrophoresis and fluorography. Major polypeptides which were resolved by this procedure were designated by number and further analyzed. Many of these major polypeptides appeared as a series of spots which corresponded to charge isomers. Two of these polypeptides (5 and 6) were shown to be acidic, glycosylated and to comprise the subunits of a dimeric protein of molecular weight 70,000. Some of the polypeptides (4a and 5a) were shown to be secreted from testicular peritubular cells which contaminated the Sertoli cell cultures. However, many of the polypeptides (1,2,3,4,5,5b and 6) were specifically secreted from the Sertoli cells. The fluorogram of the secreted polypeptides obtained from cultured Sertoli cells from 20- or 60-day-old rats were similar to each other but differed from the pattern of polypeptides which were secreted from cultures of Sertoli cells from 10-day-old rats. Polypeptide 3 was identified by immunoprecipitation as testicular transferrin and the synthesis of polypeptide 3 was stimulated when the Sertoli cells were cultured in the presence of follicle-stimulating hormone (FSH), insulin, testosterone and retinol.

INTRODUCTION

The components of the fluid within the lumen of the seminiferous tubules are apparently secreted by the Sertoli cells (Waites, 1977). This fluid has been collected by micropuncture technique and has been shown to have a composition considerably different from that of blood or of lymphatic fluid. Characterization of the seminiferous tubular fluid remains far from complete because only minute amounts can be collected by these micropuncture techniques. However, the tubular fluid has been shown to contain low concentrations of a number of proteins (Koskimies et al., 1971; Koskimies and Kormano, 1973). Because of the presence of the blood-testis barrier, some of these proteins can be assumed to be Sertoli cell products. The secretion of three specific proteins, androgen binding protein (ABP) (Fritz et al., 1974; Hagenas et al., 1975), plasminogen activator (Lacroix et al., 1977) and testicular transferrin (Skinner and Griswold, 1980) have been well documented.

Sertoli cells can be grown in culture (Dorrington and Fritz, 1975) and maintain the ability to secrete proteins. Sertoli cells in culture respond to FSH and testosterone with increased synthesis of both cellular and secreted proteins (Wilson and Griswold, 1979). FSH, testosterone, insulin and vitamin A act independently and synergistically to increase ABP and transferrin secretion in culture (Karl and Griswold, 1980; Skinner and Griswold, 1981). The maintenance of Sertoli cells in culture, therefore, provides an opportunity for the detailed study of the proteins secreted by Sertoli cells and the effects of hormones on the synthesis and secretion of these proteins.

The secreted proteins from cultured Sertoli cells obtained from 20-day-old rats have been examined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Wilson and Griswold, 1979) Seven major polypeptides were identified and designated band 1 through band 7 based upon their electrophoretic mobilities. The polypeptides ranged in molecular weight from approximately 16,000 to approximately 140,000. One polypeptide, band 3 (78,000), has been identified as an iron-binding protein and is immunologically and electrophoretically similar to rat serum transferrin (Skinner and Griswold, 1980). The other polypeptides have not yet been identified.

The resolution of individual polypeptides

Accepted February 18, 1982.
Received December 21, 1981.
[1] Reprint requests.

obtained by one-dimensional gel electrophoresis is fairly limited. The studies herein are an attempt to take advantage of the higher resolution of the two-dimensional gel electrophoresis technique of O'Farrell (1975) to further characterize the secreted proteins of cultured rat Sertoli cells and to determine the effects of hormones on their synthesis and secretion.

MATERIALS AND METHODS

Chemicals

Ampholines were obtained from Bio-Rad or LKB. Agarose and sodium dodecyl sulfate (electrophoresis purity) were from Bio-Rad. Acrylamide, N,N'-methylene bisacrylamide, N,N,N',N'-tetramethylethylenediamine, and Triton X-100 were purchased from Sigma Chemical Co. Tris and urea (ultrapure grade) were purchased from Schwartz-Mann.

All tissue culture chemicals were purchased from GIBCO. F-12 medium was mixed from a powdered formulation supplied by GIBCO and F-12 minus methionine was prepared in this laboratory. Ovine FSH (NIH-S-13) was obtained from the National Pituitary Agency. Testosterone, bovine insulin and neuraminidase (Type V) were obtained from Sigma.

[^{35}S]methionine (approximately 1000 Ci/mmol) was purchased from New England Nuclear. Rat serum transferrin and rabbit anti-rat serum transferrin were obtained from Cappell Labs.

Cell Culture

Ten-, 20-, and 60-day-old Sprague-Dawley rats were obtained from the Laboratory Animal Resources Center at Washington State University.

Sertoli cell cultures were prepared by a modification of previously described methods except that serum was not used in the media (Dorrington and Fritz, 1975; Wilson and Griswold, 1979). Peritubular cell cultures were also prepared as previously described and were subcultured in medium which contained 10% calf serum (Wilson and Griswold, 1979). All cells were plated in Linbro 24-well plates in 1 ml of Ham's F-12 medium. Hormones were administered at the following final concentrations: FSH, 25 ng/ml (NIH-S-13); testosterone, 0.7 μM; insulin, 5.0 μg/ml; retinol, 0.3 μM.

Cells were incubated for 5 days, then the medium was changed to F-12 minus methionine and 40 μCi of [^{35}S]methionine was added to each well. The cells were incubated with isotope for a 12 h period, the medium was collected, dialyzed (cellulose dialysis tubing, Van Waters and Rogers; 12,000 mol. wt. cutoff) for 60 h at 4°C, lyophilyzed and dissolved in lysis buffer (O'Farrell, 1975). Dissolved samples were either analyzed immediately or frozen at −70°C.

Immunoprecipitation

Transferrin immunoprecipitations were performed using rabbit anti-rat serum transferrin. The immunoprecipitates were washed by repeated centrifugation through a Tris/saline solution (0.05 M Tris, pH 7.4 and 0.9% NaCl) which contained 0.25 M sucrose.

Neuraminidase Treatment

Crystalline neuraminidase (0.5 mg) was dissolved in the dialyzed media from 2 culture wells and incubated for 24 h at 37°C in the presence of 0.1 M sodium acetate and 0.001 M calcium chloride (Spiro, 1962).

Two-Dimensional Gel Electrophoresis

Equilibrium two-dimensional gel electrophoresis was performed essentially as described by O'Farrell (1975) with some modifications. Samples containing from approximately 10^5 to 10^6 cpm of ^{35}S-labeled protein were applied in sample volumes up to 200 μl. Triton X-100 was substituted for Nonidet P-40 in the first-dimension gels, which were generally run for 6600 volt-hours. The first dimension of nonequilibrium gels (O'Farrell et al., 1977) were run for 1600 volt-hours. The equilibration times were reduced to 1 h and .5 h with 1 change of equilibration buffer. Five to 15% linear gradient polyacrylamide gels were used for the second dimension. Nonreducing two-dimensional gels were run identically except that 2-mercaptoethanol was deleted either from the lysis buffer or from both lysis buffer and equilibration buffer.

The pH gradients in the isoelectric focusing gels were measured by placing 5 mm gel sections into 0.5 ml of degassed distilled water. After 20 min, the pH was measured using a pH meter.

Fluorography

After electrophoresis, gels were prepared for fluorography as described by Bonner and Laskey (1974) with the following modifications: gels were fixed overnight in 10% acetic acid, 45% methanol, 45% water. Glacial acetic acid was substituted for dimethyl sulfoxide as the solvent for the diphenyl oxazole (PPO). Gels were immersed in PPO/acetic acid solution (20% w/v) for 1.5 h, then in water for 45 min.

Fluorography was done as described by Bonner and Lasky (1974) and Lasky and Mills (1975) using either Kodak XR-2, XR-5 or XAR-5 X-O-Mat film. The film was generally exposed to the dried gels for approximately 24 h.

RESULTS

Identification of Sertoli Cell-Secreted Proteins

Cultures of Sertoli cells were prepared from 20-day-old rats. The ^{35}S-labeled proteins present in the medium were analyzed by two-dimensional gel electrophoresis and visualized by fluorography. The mixture of ampholines which was used in the first dimension of the gels created a nearly linear pH gradient which extended from pH 3.5 to pH 7.7. The mixture consisted of 3 ampholines with pH limits of 3 to 10, 5 to 7 and 2.5 to 4 in a ratio of 2:1:1. A typical fluorogram from this type of analysis is shown in Fig. 1. The polypeptide spots extend from pH 3.7 to pH 7.7 and from a molecular weight of 140,000 to 12,000. The

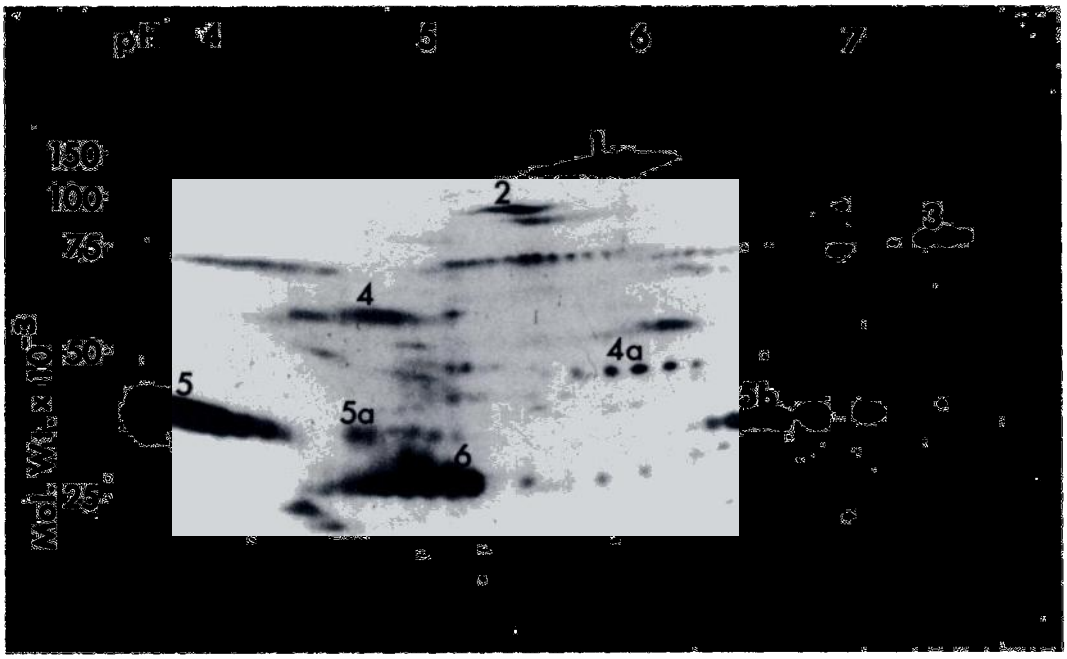

FIG. 1. A fluorogram of a two-dimensional separation of ^{35}S-proteins secreted by cultured Sertoli cells from 20-day-old rats. Prominent polypeptides which will be discussed in further analyses are numbered for reference.

major polypeptides which will be discussed in further analyses are numbered for identification. Note that the molecular weights and pH markers in Fig. 1 are approximate. Table 1 lists the average molecular weight and isoelectric points of the designated proteins obtained from the analyses of several fluorograms. In addition to these major polypeptides, many spots of lower intensity are apparent in Fig. 1. Occasionally, in this type of analysis a small shift in the pH gradient in the first dimension may cause polypeptide 3 or 5 to be lost from the second dimension.

The majority of proteins from both Sertoli cell and peritubular cell culture media displayed charge heterogeneity. This is typical of secreted glycoproteins and is thought to be due at least in part to differing amounts of sialic acid on

TABLE 1. Properties of polypeptides on fluorograms of two-dimensional gels of secreted proteins. The isoelectric point (pI) is the mean ± the standard deviation of determinations on 4 or 6 gels. If several charge isomers were present the pI is presented as a range.

Peptide number	Number of distinct charge isomers	pI	Molecular weight
1	1	6.0 ± 0.1 (6)	140,000
2	1	5.5 ± 0.2 (6)	120,000
3	3 to 4	7.2 ± 0.1 to 7.4 ± 0.2 (4)	78,000
4	1	5.1 ± 0.1 (6)	67,000
4a	4 to 5	5.9 ± 0.1 to 6.3 ± 0.1 (6)	51,000
5	6 to 8	3.7 ± 0.1 to 4.5 ± 0.1 (4)	41,000
5a	1	4.9 ± 0.1 (6)	39,000
5b	4	6.5 ± 0.1 to 7.1 ± 0.1 (6)	39,000
6	6 to 7	4.8 ± 0.2 to 5.4 ± 0.1 (6)	29,000

FIG. 2. Fluorograms of two-dimensional electrophoresis on ^{35}S-secreted proteins by cultured Sertoli cells from 20-day-old rats. *A*) Normal profile of secreted proteins. *B*) Profile of secreted proteins which were treated with neuraminidase before analysis by two-dimensional electrophoresis. *C*) Fluorogram of two-dimensional gel of secreted proteins where mercaptoethanol was deleted from only the isoelectric focusing gel (first dimension). *D*) Fluorogram where mercaptoethanol was deleted from both the isoelectric focusing gel and the polyacrylamide gel electrophoresis. All gels are arranged with the acidic region to the left. The *small bars* at the bottom of each fluorogram denote the approximate locations of pH 4, 5, 6 and 7, left to right.

the carbohydrate portions of the molecules (Anderson and Anderson, 1977). The higher molecular weight proteins showed streaking in the first dimension which could be due both to charge heterogeneity and poor solubility of these proteins. In order to determine to what extent sialic acid was present in the Sertoli cell-secreted proteins, dialyzed culture medium was incubated with neuraminidase prior to preparation for electrophoresis. The fluorogram of the two-dimensional gel of the neuraminidase-treated proteins is shown in Fig 2B. A fluorogram of an analysis of untreated proteins is shown in Fig. 2A (same as Fig. 1). Many polypeptides focused at a more basic pH as a result of the removal of sialic acid. The most striking change in isolelectric point was observed with polypeptide 6, which after neuraminidase treatment focused at a point nearly 3 pH units more basic than the most acidic variant of the intact molecule (Fig. 2A). These results suggest that this small (29,000 mol wt.) polypeptide is heavily glycosylated.

Secreted proteins were also analyzed under nonreducing conditions in order to determine the presence of polypeptides linked by disulfide bonds (Figs. 2C and D). When mercaptoethanol was deleted from the isoelectric focusing gel, polypeptides 5 and 6 focused at the same pI (Fig. 2C). When mercaptoethanol was deleted from both dimensions of the gel, both polypeptides 5 and 6 were absent and a new spot appeared at a molecular weight which was equal to the sum of the molecular weights of peptides 5 and 6 (Fig. 2D). Polypeptides 5 and 6 must compose a dimer of approximately 65,000 to 70,000 mol. wt. under nonreducing conditions. The decrease in the number of higher molecular weight proteins seen on reducing gels may be due to poor migration of these proteins into the gels. This phenomenon has been observed previously for large proteins under nonreducing conditions (Clemetson et al., 1979).

Sertoli cells prepared by our methods typically contain 5% to 10% contamination by

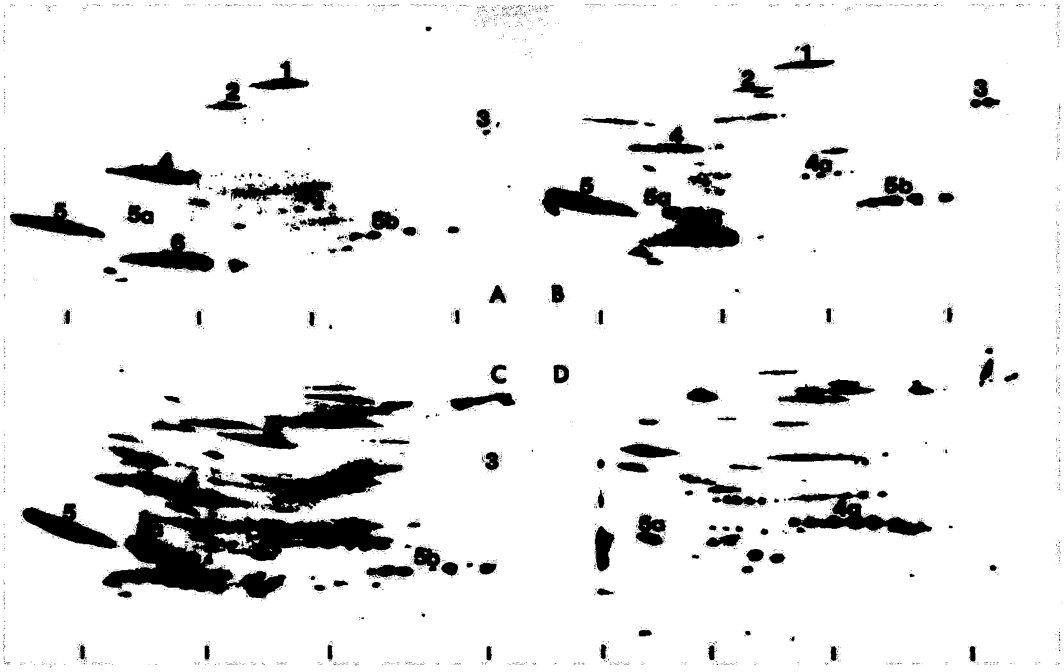

FIG. 3. Fluorograms of two-dimensional analyses of ^{35}S-secreted proteins from cultured Sertoli cells and peritubular cells from 20-day-old rats. *A*) Secreted proteins from a Sertoli cell culture selected because only a small percentage (less than 1%) of peritubular cells were present. *B*) Secreted proteins from a more standard Sertoli cell culture which contained 5–7% peritubular cells. *C*) Secreted proteins from a culture which was seeded with equal numbers of Sertoli cells and peritubular cells. *D*) Secreted proteins from subcultured peritubular cells. All gels are arranged with the acidic region to the left. The *small bars* at the bottom of each fluorogram denote the approximate locations of pH 4, 5, 6 and 7, left to right.

peritubular cells (testicular myoid cells and fibroblasts) which also secrete proteins into the culture medium (Fritz et al., 1975; Tung et al., 1975; Wilson and Griswold, 1979). At this level of contamination, major secreted proteins from peritubular cells might appear in significant amounts on two-dimensional fluorograms of the proteins in Sertoli cell culture medium. To identify these peritubular cell products, two-dimensional gels were also run on the proteins secreted from peritubular cell cultures and from mixed cultures of Sertoli cells and peritubular cells.

Figure 3A shows the fluorograph of a two-dimensional separation of proteins from the medium of a Sertoli cell culture selected for its very low level of peritubular cell contamination. This culture was judged by examination with the light microscope to contain less than 1% peritubular cells. Figure 3D shows the proteins from a peritubular cell culture which had been subcultured several times and was devoid of Sertoli cells. Figure 3B is the same as Fig. 1 and is an analysis of proteins secreted from a more typical Sertoli cell culture which contained 5–10% peritubular cells (Fritz et al., 1975). Figure 3C shows the results of a fluorograph of a two-dimensional separation of proteins which were obtained from cultures of Sertoli cells which were intentionally plated with an equal number of peritubular cells. Note that as the percentage of peritubular cells in the cultures increased, the total number of apparent polypeptides increased. In particular, polypeptides designated 4a and 5a appeared to originate from peritubular cells. Those bands which have a molecular weight equal to band 3 but are more acidic than band 3 also appear to originate from peritubular cells. Those polypeptides designated 1,2,3,4,5,5b and 6 appeared to be of Sertoli cell origin.

Sertoli cells from rats of several different ages were cultured and the secreted proteins from these cells were also labeled with [^{35}S]methionine and analyzed and compared by two-dimensional electrophoresis (Fig. 4).

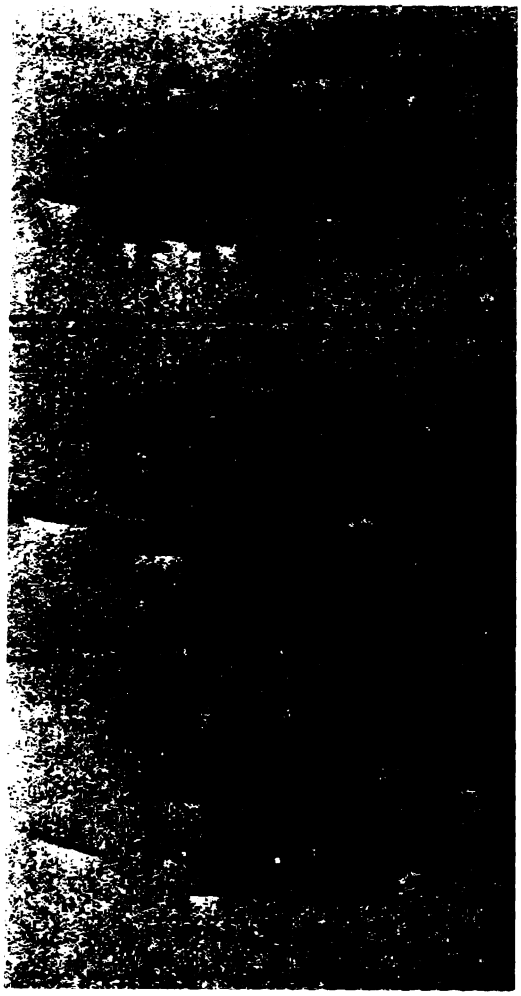

FIG. 4. Fluorograms of two-dimensional electrophoretic analysis of ^{35}S-secreted proteins obtained from cultured Sertoli cells which were obtained from rats of different ages: *A*) 10-day-old rats, *B*) 20-day-old rats, and *C*) 60-day-old rats. All gels are arranged with the acidic region to the left. The *small bars* at the bottom of each fluorogram denote the approximate locations of pH 4, 5, 6 and 7, left to right.

The pattern of polypeptides on the fluorograms were similar if the secreted proteins were obtained from animals which were 20 or 60 days of age (Figs. 4B and C). The fluorogram of proteins secreted from Sertoli cells from 10-day-old rats was different from the fluorograms of secreted proteins from the older rats (Fig. 4A). It also appears that band 4 is increased and band 5, 6 and 6b are relatively decreased in preparations from 60-day-old animals.

It was of interest to determine if Sertoli cells secreted any proteins which were more basic than band 3 protein. The secreted polypeptides from cultures of Sertoli cells from 20-day-old rats were analyzed by nonequilibrium isoelectric focusing and polyacrylamide gel electrophoresis (Fig. 5) (O'Farrell et al., 1977). In this type of analysis the basic proteins are most easily analyzed than when equilibrium isoelectric focusing is used. The polypeptides in Fig. 5 are numbered to correspond to the polypeptides in Figs. 1 through Figure 5A is a fluorograph of a nonequilibrium two-dimensional analysis of proteins secreted from cultures which contained only F-12 medium. Figure 5B is a corresponding analysis of proteins which were secreted from

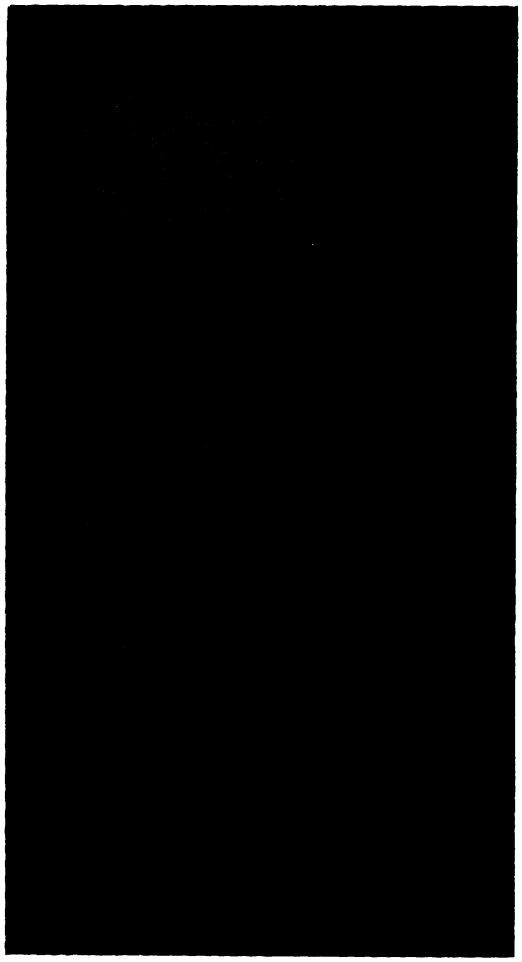

FIG. 5. Fluorograms obtained by non-equilibrium two-dimensional gel electrophoresis of secreted proteins from cultured Sertoli cells from 20-day-old rats. *A*) Control. *B*) Cultures treated with FSH, insulin, testosterone and retinol. *C*) Testicular transferrin which was immunoprecipitated from a preparation of ^{35}S-secreted proteins.

cultures which contained F-12 medium and ovine FSH-S13 (25 ng/ml), insulin (5 μg/ml), retinol (0.35 μM) and testosterone (0.7 μM). This hormone mixture has been previously shown to stimulate ABP and transferrin secretion from cultured Sertoli cells (Karl and Griswold, 1980; Skinner and Griswold, 1981). When hormones were present in the medium there was a relative increase in the amount of polypeptide 3 (Fig. 5B). The proteins shown in Fig. 5B were incubated with rabbit anti-rat transferrin and the immunoprecipitate was analyzed. Polypeptide 3 corresponded to testicular transferrin (Fig. 5C). Figs. 5A and B also show that the testicular transferrin was the most basic polypeptide present in the mixture of secreted proteins. In the preparations shown in Figs. 5A and B, proteins 2, 4 and 4a are present in low amounts but can be seen if the fluorogram is exposed for a longer time (results not shown).

DISCUSSION

When the proteins secreted by cultured Sertoli cells were analyzed by two-dimensional gel electrophoresis, a number of different proteins present in the medium could be resolved. Many of the proteins which were present on the fluorograms in relatively low abundance were also found in fluorograms of subcultured peritubular cells. However, the most abundant polypeptides in the medium from Sertoli cell cultures were not found in subcultured peritubular cell cultures. The most heavily ^{35}S-Sertoli cell-specific protein was a very acidic, 70,000 dalton glycoprotein which was composed of disulfide-linked monomers of 41,000 and 29,000 daltons (polypeptides 5 and 6). The large number of charge isomers and the electrophoretic properties of the monomers suggested that the native protein must be heavily glycosylated. In addition, this protein stains poorly with Coomassie blue on acrylamide gels but can be easily stained with reagents which are specific for sugars on glycoproteins (unpublished observations).

It has been demonstrated that the secretion of ABP and transferrin is under hormone regulation in cultured Sertoli cells (Karl and Griswold, 1980; Skinner and Griswold, 1981). Maximum amounts of transferrin can be detected in the medium of cells which have been maintained in the presence of FSH, insulin, testosterone and retinol (Skinner and Griswold, 1981). The increased synthesis of testicular transferrin (polypeptide 3) in these hormone-treated cultures can easily be seen in the increased relative radioactivity associated with polypeptide 3. The amount of ABP synthesized under the same conditions is much less than the amount of transferrin (Skinner and Griswold, 1981). ABP is reported to have 2 subunits of 45,000 and 41,000 daltons which have isoelectric points which range from 4.7 to 5.5 (Musto et al., 1980). No major polypeptide can be detected on the fluorograms of the Sertoli cell-secreted proteins which corresponds to these molecular parameters. Minor spots which may correspond to ABP subunits were detected on fluorograms which were overexposed. The synthesis of some of the other major Sertoli cell-secreted glycoproteins, most notably polypeptides 5 and 6, did not appear to be greatly influenced by the presence or absence of hormones in the medium.

It has been suggested previously that polypeptide 1 is ceruloplasmin (Skinner and Griswold, 1980). This tentative identification was based solely on the molecular weight of this protein and the presence of similar immunoreactive protein in serum. In addition, protein 1 has an isoelectric point (Table 1) which is similar to that reported for rat ceruloplasmin (Anderson and Anderson, 1977). No additional information about the possible structure or function of polypeptides 2, 4 or 5B is available. The amount of radioactivity in polypeptide 4 seemed to be somewhat variable from different preparations of secreted proteins.

Polypeptides 4A and 5A appeared to be products of peritubular cells. Both of these peptides increased relative to Sertoli cell specific proteins in preparations which contained greater numbers of peritubular cells and both of these polypeptides were present in preparations from purified subcultured peritubular cells. The presence of these two polypeptides can serve as a convenient and sensitive assay for the presence of peritubular cells in primary Sertoli cell cultures. A close examination of the fluorograms in Fig. 3 will reveal several other polypeptides that could be utilized as indicators of the presence of peritubular cells in the cultures.

When the secreted glycoproteins from Sertoli cell cultures prepared from rats of 20 days of age or 60 days of age were analyzed by the two-dimensional gel electrophoresis, the patterns of polypeptides on the fluorogram were not significantly different. By 10 days of age in the rat, the blood testis barrier has not

formed and meiosis has not been initiated. However, by 20 days of age in the rat, meiosis has been initiated and a lumen has been formed in the tubule and in a morphological sense, the testis has matured (Vitale et al., 1973). The polypeptide patterns of the two-dimensional analysis of the proteins secreted from Sertoli cells prepared from 10-day-old animals differed greatly from that prepared from the older animals. Many of the same major polypeptides were present in lower relative concentration (polypeptides 5 and 6) while many additional polypeptides were also present. It is important to note, however, that the cultures of Sertoli cells from 10-day-old rats have not been well characterized and it was more difficult in these cultures to microscopically determine the relative number of peritubular cells.

ACKNOWLEDGMENTS

The authors thank Alice Karl for cell culture assistance and Holly Schumacher for help in preparing this manuscript. The FSH used in this study was obtained from the National Pituitary Agency. This work was supported by Grants HD 10808 and HD 00253 from the National Institute of Child Health and Human Development.

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