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Abstract

Murine adrenal tumor cells (Y-1 clone) were stimulated by adrenocorticotropic hormone (ACTH) and cyclic adenosine 3',5'-monophosphate (cyclic AMP) to produce steroid hormone (delta 4, 3-keto steroids). The steroids were secreted into the medium immediately after synthesis. The optimum concentrations of ACTH and cyclic AMP for stimulation of steroid production were 10(-2) U/ml and 1.0 mM, respectively. In serum-free medium, ACTH and cyclic AMP stimulated steroidogenesis in Y-1 cells, but the amount of steroid hormone in the culture medium was low. However, a high level of steroid production was maintained with medium containing 10 mg/ml bovine serum albumin (BSA). In culture medium containing a higher concentration of BSA, Y-1 cells did not become spherical as is usually the case when steroid production is stimulated by ACTH or cyclic AMP. The morphological changes did not always correlate with steroid secretion by Y-1 cells.

KEYWORDS: Y-1 clone, steroid hormone, ACTH, cyclic AMP

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Composition of Culture Media for Steroid Hormone Secretion by Murine Adrenal Tumor Cells, Y-1 Clone

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Murine adrenal tumor cells (Y-1 clone) were stimulated by adrenocorticotropic hormone (ACTH) and cyclic adenosine 3′, 5′-monophosphate (cyclic AMP) to produce steroid hormone (delta 4, 3-keto steroids). The steroids were secreted into the medium immediately after synthesis. The optimum concentrations of ACTH and cyclic AMP for stimulation of steroid production were 10^{-2} U/ml and 1.0 mM, respectively. In serum-free medium, ACTH and cyclic AMP stimulated steroidogenesis in Y-1 cells, but the amount of steroid hormone in the culture medium was low. However, a high level of steroid production was maintained with medium containing 10 mg/ml bovine serum albumin (BSA). In culture medium containing a higher concentration of BSA, Y-1 cells did not become spherical as is usually the case when steroid production is stimulated by ACTH or cyclic AMP. The morphological changes did not always correlate with steroid secretion by Y-1 cells.

Key words : Y-1 clone, steroid hormone, ACTH, cyclic AMP

The tissue culture of hormone-secreting adrenal tumors has been reported (1-6). It has been shown that these cultures are stimulated by adrenocorticotropic hormone (ACTH) and cyclic adenosine 3′, 5′-monophosphate (cyclic AMP)(4, 5) to secrete increased amounts of steroid hormone. The murine adrenal tumor cell line, Y-1, established by Yasumura *et al.*(7), has epithelial morphology and a striking similarity to the normal adrenal cortex in its biological function. Y-1 cells have produced delta 4, 3-keto steroids in response to ACTH and cyclic AMP for several years in continuous culture (7).

Furthermore, stimulation of Y-1 cells with ACTH induces secretion of steroids into the medium, concurrently with a change in cellular morphology from polygonal to round cells (7). It is of interest to study the interrelationship between cell function and cell morphology. In the present study, the effect of the culture medium composition, such as the concentration of serum and bovine serum albumin (BSA), on the ability to secrete steroids and on morphological changes in Y-1 cells was investigated.

Materials and Methods

Cells and culture conditions. The ACTH-sensitive Y-1 murine adrenal tumor cell line was supplied by Dr. T. Uchida, Department of Pathol-

ogy, Nippon University. The Y-1 cells were maintained in 25-cm^2 tissue culture flasks in Ham's F-12 medium (Grand Island Biological Co., Grand Island, NY, USA) containing 10% newborn bovine serum (Nakashibetsu Serum Center, Nakashibetsu, Hokkaido, Japan). Cells were subcultured by rinsing with Ca^{2+} and Mg^{2+}-free phosphate-buffered saline [PBS(−)] and incubated at 37°C with 0.25% trypsin for 5 min. After incubation, complete medium was added, and the cells were suspended and distributed into plastic petri dishes or bottles. Culture medium was changed three times a week.

Human diploid cells, HAIN-44 and HAIN-55, which were established from fetal lung fibroblasts by Okumura *et al.* (8), were used. These normal cells were maintained in Eagle's basal medium (Grand Island Biological Co.) supplemented with 10% newborn bovine serum.

Stimulation of secretion of steroid hormone. Cells were incubated in 35-mm plastic petri dishes at a plating density of 2 to 3×10^5 cells per dish at 37°C in a moisture-saturated atmosphere of 5% CO_2. A fully confluent monolayer culture was obtained after 4 to 5 days' incubation. The ACTH used was ACTHER (Armour, Phoenix, Ariz, USA). Cyclic AMP and dibutyl cyclic AMP (db-cyclic AMP) were supplied by the Yamasa Shoyu Co., Ltd., Choshi, Japan. BSA was purchased from Wako Pure Chemical Ind., Ltd., Osaka, Japan. Cells in confluent monolayer were exposed to ACTH and other reagents in growth medium for a given period of time at 37°C. After incubation, medium was collected, by centrifugation (2,000 rpm, 10 min) and subjected to the steroid assay.

Steroid assay. The amount of delta 4, 3-keto steroids in culture medium was determined according to Silber *et al.* (9) and Sayers *et al.* (3) with the following modifications : (a) the alkaline washing after methylene chloride extraction of steroids was omitted ; (b) an aliquot of methylene chloride extract was allowed to react with 30 NH_2SO_4 while vigorously mixing on a Vortex mixer for 20 to 30 sec ; and (c) fluorescence of the H_2SO_4 layer was measured with an exciting wavelength of 470 nm and an emitting wavelength of 535 nm. Cell sheets were washed twice with PBS(−) and harvested by scraping them with a rubber policeman. The cell suspension was centrifuged at $500\times g$ for 10 min, and the pellet was dissolved in 0.5 N NaOH by incubating overnight. The amount of protein was determined by the method of Lowry *et al.* (10). The results were expressed as μg of delta 4, 3-keto steroids per mg protein released in the culture medium during the incubation period.

Results

Inoculation of Y-1 cells at a density of 2×10^5 cells per 35-mm dish resulted in an almost confluent state of cells (8.8×10^5) within about 5 days. Throughout the stationary phase, Y-1 cells secreted delta 4, 3-keto steroids, the levels of which were increased by incubation with ACTH. The stimulation

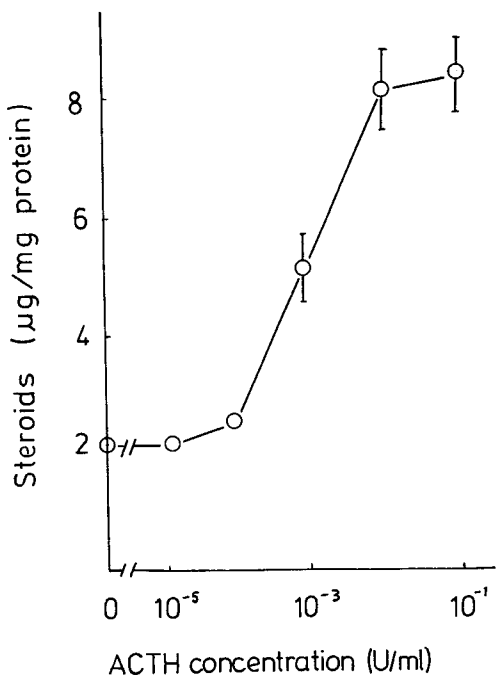

Fig. 1 Relation of steroid production by Y-1 cells to the concentration of ACTH in the medium. Cells were inoculated at 2×10^5 cells per 35-mm plastic petri dish in F-12 medium supplemented with 10% serum. Nearly confluent cells obtained after 5 days of culture were used for steroid stimulation. ACTH was added to culture medium containing 10% serum, and after 24 h, the culture fluid was collected for assay of steroids.

of steroid production in Y-1 cells depended on the concentration of ACTH (Fig. 1). The maximum steroidogenic response to ACTH stimulation in these cells was observed at 10^{-2} U/ml of ACTH. Changes with time in the secretion of steroids by Y-1 cells into the medium are shown in Table 1. The amount of steroids secreted in medium containing ACTH at 2 h of incubation was 2.09 μg/mg protein. The ratio of steroid production in medium with ACTH to that without ACTH reached 26.1 at 2 h of incubation, but this ratio decreased with longer incubation time. Thus, it was found that 2 h of incubation with ACTH is sufficient to stimulate steroid secretion.

The maximum secretion of steroids was attained at 1 mM of both cyclic AMP and db-cyclic AMP. Fig. 2 illustrates that at this concentration they stimulated steroidogenesis to the same extent as 10^{-2} U/ml of ACTH. After the addition of cyclic AMP and db-cyclic AMP to the medium, the beginning of steroid secretion was slightly delayed, but steroid secretion increased linearly as the same as ACTH for 24 h.

The human diploid cell lines HAIN-44 and HAIN-55 were used to examine steroidogenesis and the morphological changes in-

Table 1 Secretion of steroids by Y-1 cells incubated with or without ACTH

Incubation time (h)	Steroids in medium[a]		Ratio (b/a)
	Without ACTH (a)	With ACTH (10^{-2}U/ml) (b)	
2	0.08	2.09	26.1
4	0.21	2.83	13.2
12	0.66	4.41	6.7
24	1.78	8.35	4.7

a: μg/mg protein.

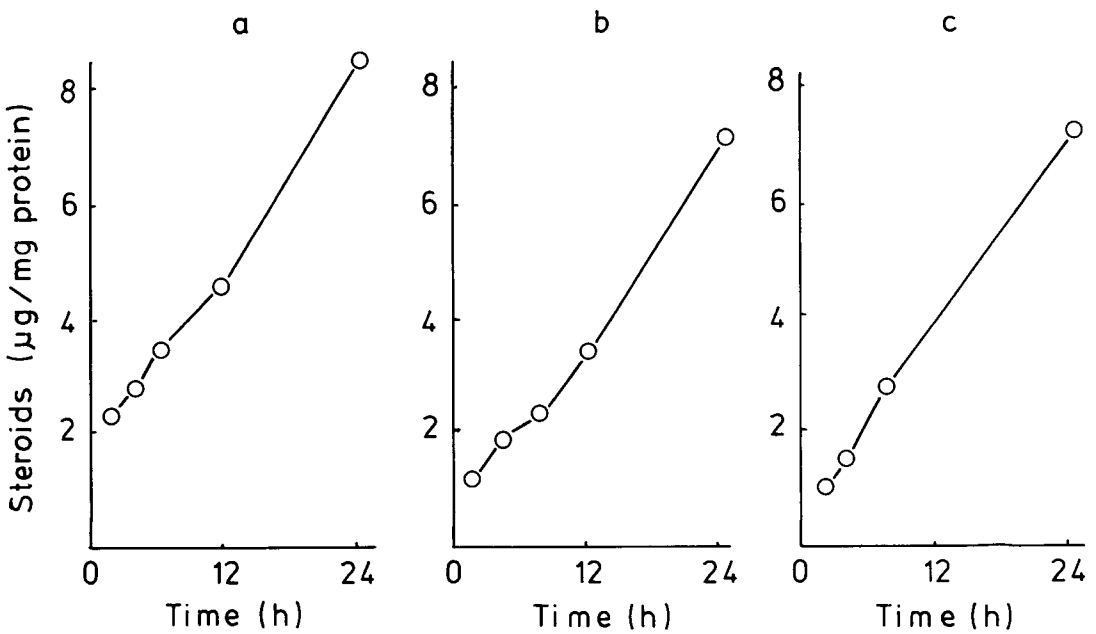

Fig. 2 Time course of steroid production by Y-1 cells stimulated by ACTH, cyclic AMP and dibutyryl cyclic AMP. Cells grown for 5 days in medium containing 10% serum were used for steroid stimulation. Reagents including ACTH were added to medium containing 10% serum. At various times of incubation, the culture fluid was collected and steroids were assayed. a : ACTH 10^{-2} U/ml, b : cyclic AMP 1 mM, c : dibutyryl cyclic AMP 1 mM.

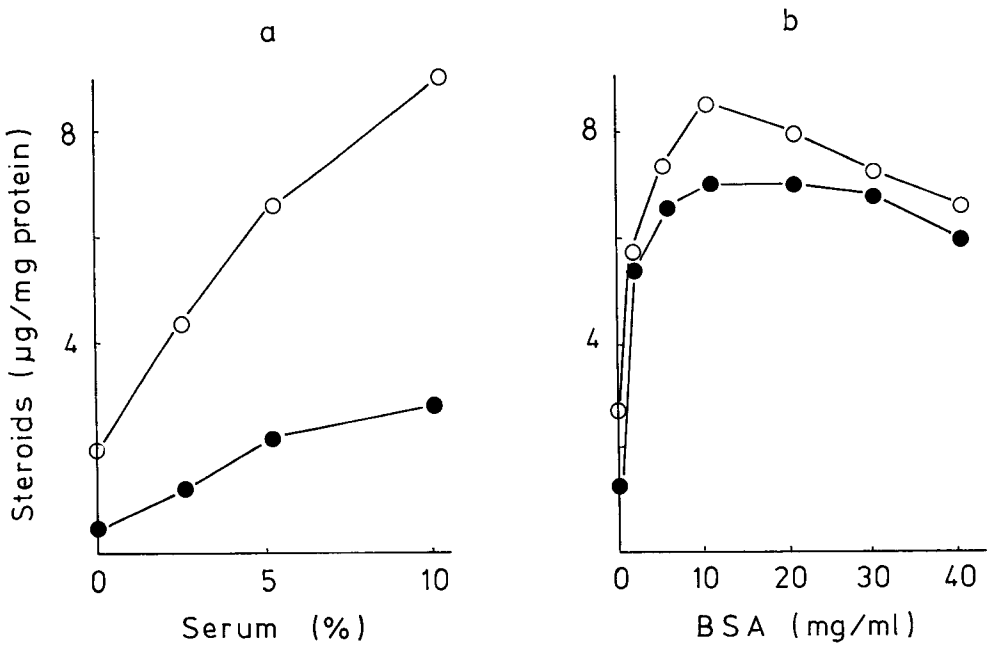

Fig. 3 Effect of serum or BSA present in the medium on steroid production by Y-1 cells. Cells were cultured for 5 days in medium containing 10% serum. Then, the stimulation of steroid production by ACTH was assayed in medium supplemented with various concentrations of serum (Fig. 3-a) or BSA (Fig. 3-b). The culture fluid was collected after 24 h of incubation, and steroids were assayed. ○——○, ACTH at 10^{-2} U/ml ; ●——●, no ACTH.

duced by ACTH and cyclic AMP in cells other than Y-1. HAIN-44 and HAIN-55 confluenced within 5 days of being inoculated at a density of 2×10^5 cells per 35-mm dish. Addition of ACTH (10^{-2} U/ml) or db-cyclic AMP (1 mM) to these cells during the stationary phase resulted in neither steroidogenesis nor morphological changes.

Stimulation of glucocorticoid synthesis by ACTH requires Ca^{2+}, and the steroidogenic effect of cyclic AMP depends on intracellular Ca^{2+} (11). Thus, steroidogenesis induced by addition of Ca^{2+} to F-12 medium was examined. It was found that the medium was sufficient for steroidogenesis without additional Ca^{2+} (data not shown).

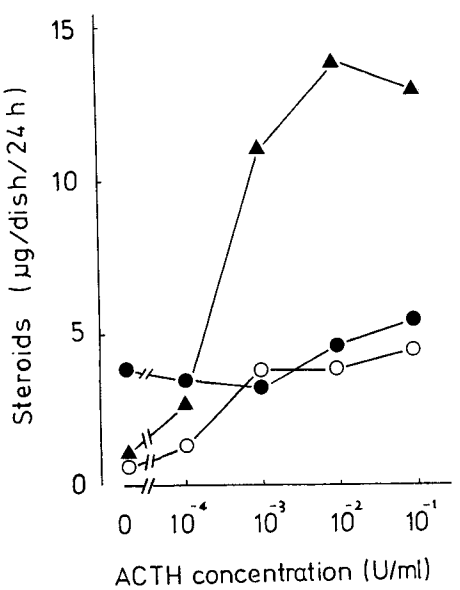

Fig. 4 Effect of different compositions of culture media on steroid production by Y-1 cells. After the cells were cultured for 5 days in medium with 10% serum, they were treated with 10^{-2} U/ml ACTH for 24h in different culture media. Then, the culture fluid was collected, and steroids were assayed. The results are expressed as μg steroid/dish. ○——○, F-12 medium ; ▲——▲, F-12 medium plus 10% serum ; ●——●, F-12 medium plus BSA 80 mg/ml.

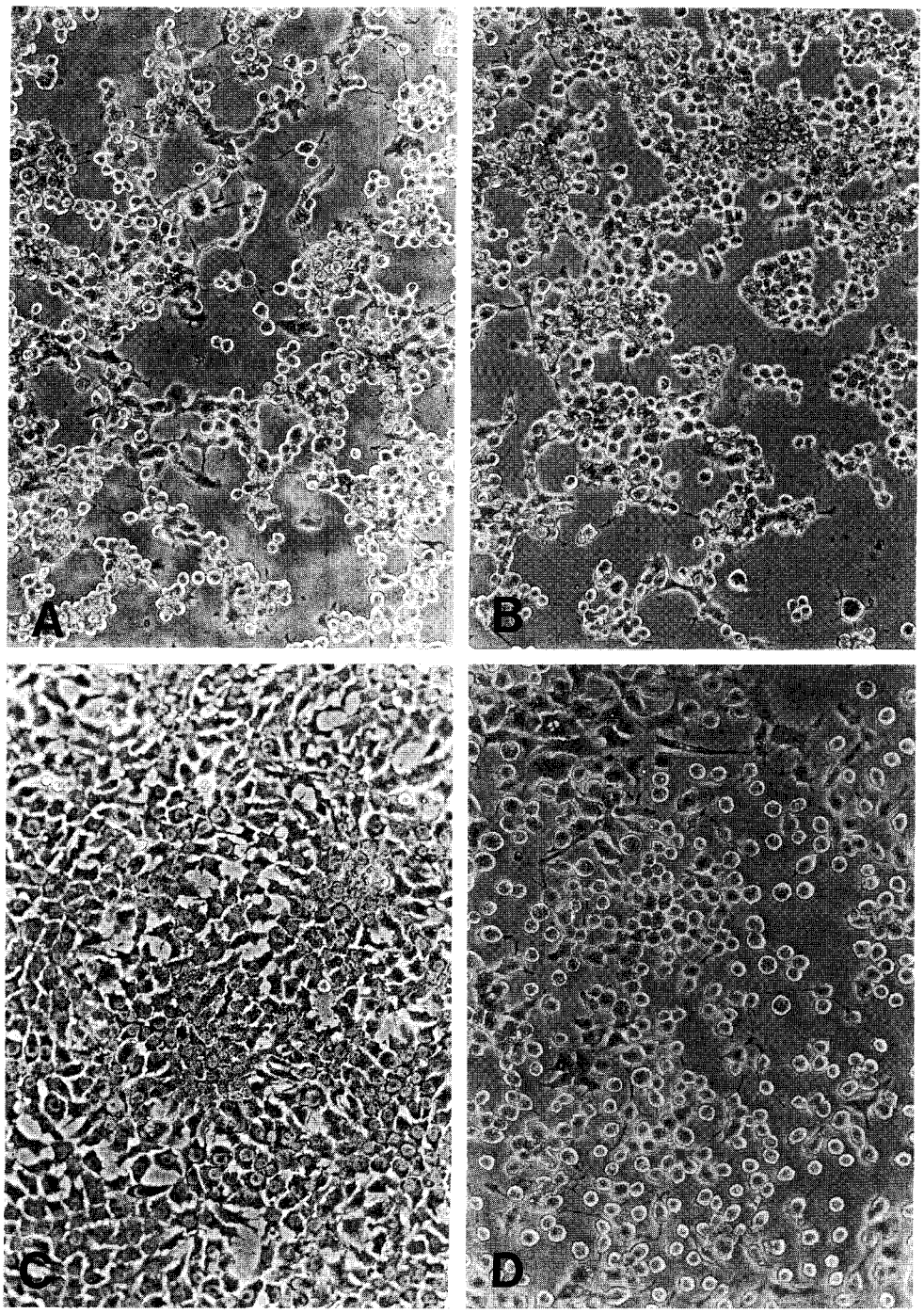

Fig. 5 Phase contrast micrographs of Y-1 cells. Cells were treated with ACTH 10^{-2} U/ml for 24 h in F-12 medium with no additions (A), or containing 10% serum (B), 80 mg/ml BSA (C), or 80 mg/ml PVP (D).

The effect of the serum concentration of the medium on steroid secretion was studied. As shown in Fig. 3-a, steroidogenesis was stimulated nearly 4 times by ACTH, but the ratio of stimulation by ACTH was the same when the serum concentration of the medium was 2.5%, 5% and 10%. Since serum in medium stimulates production of steroids, BSA was added to the medium instead of whole serum. As Fig. 3-b shows, the maximum steroid secretion was observed with ACTH when 10 mg/ml of BSA was present. The steroid production gradually increased, without ACTH in the medium, according to the serum concentration (Fig. 3-a). Furthermore, much more steroid was secreted into medium without ACTH when BSA was added to the medium (Fig. 3-b). However, when the BSA concentration of the medium was 80 mg/ml, the stimulation of steroid secretion by ACTH was not observed (Fig. 4).

A high molecular weight polymer compound, polyvinyl-pyrolidone (PVP), was used instead of BSA, but no additive effect on steroid secretion was seen at various concentrations of PVP in the medium (data not shown).

Phase contrast microphotographs of Y-1 cells treated by ACTH (10^{-2} U/ml) in media of various compositions are shown in Fig. 5. Monolayer cultures underwent a remarkable morphological change from flattened to spherical cells after ACTH stimulation in medium containing 10% serum (Fig. 5-B). At a high concentration of BSA (80 mg/ml), Y-1 cells showed no morphological change. In contrast, most of Y-1 cells which were cultured in medium containing neither serum nor BSA became more spherical, although the cells secreted only a small amount of steroid as when incubated in medium with 80 mg/ml BSA (Figs. 5-A, 5-C). Y-1 cells also became spherical after they were incubated in medium containing PVP in spite of secreting only a small amount of steroid (Fig. 5-D).

Discussion

Y-1 cells of murine adrenal tumor origin were induced to secrete more steroids by the addition of ACTH or cyclic AMP to the medium. ACTH is known to elevate the intracellular cyclic AMP concentration. A high level of cyclic AMP stimulates various intracellular protein kinase activities, and, in turn, the intracellular concentration of NADPH, which is required for hydroxylation reactions involved in steroidogenesis, is elevated and consequently promotes steroidogenesis (5). Based on the mechanism of the action of ACTH, steroid secretion by the addition of cyclic AMP may be started earlier than ACTH. In our study, steroid secretion induced by ACTH began slightly earlier than that induced by cyclic AMP and db-cyclic AMP, although similar rates of stimulation were observed. This result suggests that it may take time for cyclic AMP to cross the plasma membrane and accumulate in the cytosol.

There are many biologically active constituents in serum which is added to medium, but all their functions are not clearly defined yet . After being transferred to a medium with a low serum content, Y-1 cells lose the ability to secrete steroid hormones (7). On the other hand, steroid production correlates to the concentration of BSA in culture medium (6). In our study (Fig. 3), the maximum amount of steroid secretion was obtained when 10% serum or 10 mg/ml BSA was added to the medium. However, at a higher concentration of BSA in the culture medium, the steroid secretion of Y-1 cells was not stimulated by ACTH (Figs. 3-b, 4). The ability to secrete steroid was enhanced by BSA, but the action of ACTH was suppressed by BSA at high concentrations.

Therefore, BSA is not suitable for experiments on steroid stimulation. It is probable that most of the BSA molecules were attached to the plasma membrane and bound to ACTH in the culture medium, so that ACTH could not bind to cell surface receptors.

In our experiments, the phenomenon of cells becoming spherical, which accompanies steroidogenesis, was usually observed upon the addition of either ACTH or cyclic AMP. Also, even without addition of either ACTH or cyclic AMP to the medium, similar cellular morphological changes occurred. It has been reported that there is a relation between the morphological changes and steroid secretion (7). However, our results did not indicate that cellular morphological changes are always related to steroid secretion (Fig. 5). For example, in medium containing 10% serum, spherical cells increased in proportion to the amount of steroids secreted in the medium. In contrast, cells cultured in medium with a high concentration of BSA (80 mg/ml) did not become spherical, in spite of steroid secretion. On the other hand, when the cells were cultured in F-12 medium without serum or BSA, most of the cells became spherical, but secreted only a small amount of steroid (Fig. 4). Also, as shown in Fig. 5-D, when PVP was added to medium at a high concentration (80 mg/ml), cells became spherical in the presence of ACTH, in spite of little steroid secretion. The reason why a high concentration of BSA inhibits cells from becoming spherical is not clear.

The results of the present study indicate that in medium adequate for steroid secretion, a relationship exists between the number of spherical cells and the amount of steroid secreted into the medium. However, when Y-1 cells are cultured in media of various compositions, the phenomenon of becoming spherical dose not always correlate to steroid secretion.

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