SEPARATION OF RABBIT ADRENAL CELLS BY SEDIMENTATION AT A LINEAR GRADIENT OF ALBUMIN CONCENTRATION

Kiichiro Abe

Department of Physiology, Nagasaki University School of Medicine, Nagasaki 852, Japan

Abstract The isolated cells of rabbit adrenal glands were separated by the method of sedimentation at a linear gradient of albumin concentration and incubated with $^{14}$C-cholesterol. The corticosterone and cortisol produced by the separated cells were fractionated by thin-layer chromatography and the radioactivities in these steroids were measured. The more rapidly sedimenting cells showed a greater ratio of cortisol production to corticosterone production than the more slowly sedimenting cells. This phenomenon is not due to the decrease of corticosterone production per cell by the former, but to the increase of cortisol production per cell. These results suggest that the rabbit adrenal cortex may be composed of the cells that produce corticosterone and cortisol in different ratio, i.e., physiologically heterogeneous cells.

The functional specificity on different cellular zones of the adrenal cortex in many mammals was confirmed by a number of workers, that is, aldosterone and a part of corticosterone was produced by the zona glomerulosa, and corticosterone and cortisol by both the zona fasciculata and reticularis (Gioude et al., 1956; Stachenko and Gioude, 1959). However, it has not been evident whether corticosterone and cortisol are produced by the same cell or different cells. In an attempt to elucidate this problem, the separation of rabbit adrenal cells was carried out in the present study by the method of sedimentation at a linear gradient of albumin concentration.

MATERIALS AND METHODS

Isolation of adrenal cells. Bilateral adrenal glands from a 30–40-day-old male rabbit were used to prepare cell suspensions because they produced both corticosterone and cortisol and were easily isolated by the method described below.
All glassware and gauze were pretreated with dimethyl polysiloxane (Fuji Systems Corp.). The cell suspensions were prepared by a modification of the methods of SAYERS et al. (1971). Briefly, the modified methods were as follows. The adrenals were cut into pieces and suspended by agitating with a glass paddle at 450 rpm for 15 min in 4 ml of 0.2% trypsin (Worthington Biochemical Corp.). The cell suspensions from five 15 min-dispersions were filtered through 2 sheets of gauze and the filtrate was centrifuged at 100 × g for 30 min at 4°C. The pellet (isolated cells) was washed with 10 ml of Krebs-Ringer bicarbonate buffer solution containing 0.2% glucose (KRBG) and then with 4 ml of 0.9% NaCl containing 0.1% lima bean trypsin inhibitor (LBI) and 0.01% disodium ethylenediaminetetra-acetate (EDTA). The cells were resuspended in 0.7 ml of the latter medium. The cell suspension containing about 2 × 10⁶ cells/ml was used to separate adrenal cells.

**Generation of an albumin column and separation of adrenal cells.** The present method was modified from that developed by PETERSON and EVANS (1967) for the separation of bone marrow cells, i.e., the method of sedimentation at unit gravity. A gradient was generated with the apparatus shown in Fig. 1, i.e., two 6 ml-beakers and a column (15 × 115 mm). The beakers contained 5 ml of either 0.5% bovine serum albumin (BSA) (Fig. 1A) or 6% BSA (Fig. 1B) in 0.9% NaCl containing 0.01% EDTA. The tubing (Fig. 1D) connecting two beakers was filled with 6% BSA solution. The teflon tubing (Fig. 1E) connecting beaker A (Fig. 1A) and a column (Fig. 1C) was filled with 0.5% BSA solution. While the

![Fig. 1. Apparatus for sedimentation of rabbit adrenal cells at a linear gradient of albumin concentration. Beaker A and B contain 5 ml of 0.5% and 6.0% BSA, respectively, in 0.9% NaCl containing 0.01% EDTA. Gradient solution is flowed into a glass column (C) by lowering it during mixing with a magnetic stirrer (F).](image-url)
solution in beaker A (Fig. 1A) was mixed by a magnetic stirrer (Fig. 1F), the column was carefully lowered for 30 min in order to flow the gradient solution into the column. The column of a linear gradient of 0.5–6.0% BSA was produced and then kept in an ice-cold water bath. The cell suspension was carefully layered on the top of the column. After the column was allowed to stand for 90 min in an ice-cold water bath, six successive 1.5 ml-cell fractions were collected in centrifuge tubes from the bottom of the column. In this paper increasing fraction number indicates the fractions composed of the more slowly sedimenting cells.

Cell incubation and corticosteroid assay. The cells in each fraction were washed twice with 8 ml of KRBG and then resuspended in 1 ml of KRBG containing 0.5% BSA, 0.2% LBI and 7.65 mm CaCl₂. These cell suspensions were transferred into the incubation vials containing 0.2 μCi of ¹⁴C-cholesterol (49.4 mCi/mmol) and 1.0 mU of ACTH (Armour). Cell counts in each fraction were made with a hemocytometer.

After incubation at 37°C for 2 hr under 95% O₂: 5% CO₂, 7 μg of each corticosterone and cortisol were added to each incubation medium as a carrier. The incubates were washed with 3 ml of trimethylpentane in order to remove cholesterol and then 5 ml of dichloromethane was added. The mixtures were vigorously shaken and filtered through Whatman filter 1PS (phase separating paper). The corticosteroids in the filtrates were fractionated by thin-layer chromatography on silica gel (Wakogel B-5F). The chromatogram was developed in the first dimension with a solvent mixture of chloroform and methanol in a ratio of 94: 6 at vol. (Hirose, unpublished method) and in the second dimension with a solvent mixture of ethyl acetate, n-hexane, ethanol and acetic acid in a ratio of 73: 14: 3: 10 at vol. The spots of corticosterone and cortisol were detected under ultraviolet light (254 nm). Each corticosteroid was eluted from the spots with 5 ml of ethyl acetate and the radioactivities in these eluates were measured. Nearly equivalent amounts of corticosterone and cortisol were removed from incubation medium by the extraction procedures. The recovery was approximately 90%. Each radioactivity was taken as the production of each corticosteroids.

Control experiments. Non-separated isolated-adrenal cells were treated for 90 min in an ice-cold water bath with the cell-free albumin solution fractionated from a linear gradient of albumin column by a method similar to that used in cell separation. These cells were treated in the same way as that used in separation-experimental groups and the radioactivities incorporated into each corticosteroid spot were measured.

RESULTS AND DISCUSSION

In each fraction of separated cells, the average numbers of cells, the average radioactivities incorporated into corticosteroids and that per cell are shown in
Table 1. Large differences in corticosterone production per cell were not observed among the fractions, but the cortisol production per cell by the more rapidly sedimenting cells were greater than that by the more slowly sedimenting cells. However, since these values were variable among the experiments, corticosterone and cortisol productions were expressed as percentage for each experiment. The mean percentages of each corticosteroid production from 6 experiments are shown in Fig. 2A.

In fractions 1 and 2, non-significant difference was observed between corticosterone and cortisol productions. However, corticosterone production was significantly higher than cortisol production in fraction 3 (P<0.05), 4 (P<0.01), and 5 and 6 (P<0.001). Furthermore, the percentage of cortisol production in
fractions 1 and 2 was significantly higher ($P<0.05$) than that in fractions 5 and 6. In the most rapidly sedimenting cells, i.e., in fraction 1, cortisol production was approximately equal to corticosterone production. Thus the more rapidly sedimenting cells showed a higher percentage of cortisol production than the more slowly sedimenting cells. This phenomenon is not due to the decrease of corticosterone production per cell by the former, but to the increase of cortisol production per cell (Table 1). This fact suggests that those cells are not damaged during isolation of cells. However, cytological studies in each fraction should be carried out hereafter.

These results suggest that the rabbit adrenal cortex may be composed of physiologically heterogeneous cells. However, it is possible that the ratio of cortisol production to corticosterone production may be influenced by pretreating the cells with different concentrations of albumin before incubation. In order to examine this possibility, control experiments were carried out. The average radioactivities per $10^5$ cells incorporated into corticosterone and cortisol were hardly different among the fractions, and were approximately 278 and 114 cpm/$10^5$ cells, respectively. However, since these values were variable among the experiments, the productions of each corticosteroid were expressed as percentage in a similar manner as that of separation experiments. These results are shown in Fig. 2B. In all the fractions production of corticosterone was greater ($P<0.001$) than that of cortisol. The cortisol/corticosterone ratio averaged 0.36 and non-significant difference was observed among the fractions. This value is in the range of level of the cortisol/corticosterone ratio in the peripheral plasma of rabbits (GANJAM et al., 1970; VAN DER VIES, 1961). These results show that the ratio of cortisol to corticosterone produced by non-separated cells was not influenced by pretreating the cells with different concentrations of albumin. Furthermore, in fraction 1 the percentage of cortisol production by separated cells was significantly higher ($P<0.005$) than that by non-separated cells. It could not be clarified from the present results whether corticosterone and cortisol were produced by the same cell or different cells. It is, however, suggested that even if these two kinds of corticosteroids are produced by the same cell, the ratio of productions of those may be different in each cell, that is, the rabbit adrenal cortex may be composed of physiologically heterogeneous cells.

The author is grateful to Professor T. Suzuki for his sustained interest and advice in the course of the present investigation.

REFERENCES


