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Establishment and Characterization of a Human Adrenocortical Carcinoma Cell Line That Expresses Multiple Pathways of Steroid Biosynthesis[1]

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ABSTRACT

We established a continuous cell line, NCI-H295, from an invasive primary adrenocortical carcinoma. The cell line was established in a fully defined medium (HITES) and later could be adapted for growth in a simple medium supplemented only with selenium, insulin, and transferrin and devoid of serum, steroids, fibroblast growth factor, and a source of exogenous cholesterol. NCI-H295 cells had a relatively long population doubling time and were tumorigenic when inoculated s.c. into athymic nude mice. The cultured cells had ultrastructural features of steroid-secreting cells and contained complex cytogenetic abnormalities including the presence of multiple marker chromosomes. Steroid analyses (radioimmunoassays and mass spectrometry), performed 7 to 9 years after culture initiation, demonstrated secretion of more than 30 steroids characteristic of adrenocortical cells. Total unconjugated steroid secretion in serum-supplemented medium was 2.83 $\mu g/10^6$ cells/24 h and about 4-fold less in serum-free medium. The major pathway of pregnenolone metabolism in NCI-H295 cells is androgen synthesis, with formation of dehydroepiandrosterone, androstenedione, testotesterone, and at least three sulfated androgens, as well as estrogens. In addition, formation of cortisol, corticosterone, aldosterone, and 11β-hydroxyandrostenidione indicated the presence of 11β-hydroxylase. Thus, multiple pathways of steroidogenesis are expressed by NCI-H295 cells, including formation of corticosteroids, mineralocorticoids, androgens, and estrogens. Our findings indicate the presence in NCI-H295 cells of all of the major adrenocortical enzyme systems, including 11β-hydroxylase, desmolase, 21α-hydroxylase, 17α-hydroxylase, 18-hydroxylase, lyase, sulfokinase, and aromatase. The NCI-H295 cell line should prove of value in studying the regulation, metabolic pathways, and enzymes involved in steroid formation and secretion. In addition, it may provide insights into the biology and treatment of adrenocortical carcinoma.

INTRODUCTION

Multiple pathways of steroidogenesis are present in the normal adrenal cortex, primarily involving formation of glucocorticoids, mineralocorticoids, and weak androgens (1, 2). In addition, small amounts of estrogens are secreted, and some progesterone is produced as an intermediary in the formation of other steroids. One or more of the steroid pathways may be expressed by benign adenomas and malignant carcinomas of the adrenal cortex. The former are a common finding at autopsy, especially in the elderly, and most are nonfunctioning. In contrast, carcinomas are exceedingly rare neoplasms, whose incidence has been estimated to be 0.5 to 2 per million per year (3, 4). Most carcinomas are functioning, with about 50% associated with Cushing's syndrome, 20% with virilization, 4% with both, 12% with feminization, and 4% with hypermineralocorticoidism (due to hypersecretion of aldosterone or of other salt-retaining steroids) (4). Thus only about 10% of carcinomas are nonfunctioning, and even some of these may be associated with secretion of clinically inapparent precursor steroids such as pregnenolone or 17-hydroxypregnenolone.

The sex distribution of adrenocortical carcinomas is equal, although more women have functioning tumors. While the average age at presentation is 40 to 50 years, there is a broad spectrum. Many tumors are large (with a mean diameter of 16 cm) at presentation. Areas of cystic necrosis, hemorrhage, calcification, mitoses, and local invasion are common. Seventy % of carcinomas at presentation have extraadrenal spread or distant metastases, especially to the lungs and liver.

Cultures of the adrenal cortex offer useful models to study the biology of the various steroid pathways, including their hormonal control, interrelationships, and secretion. Many studies have utilized short term cultures of normal or neoplastic human adrenal cells for these purposes (5–8). However, the list of permanent cell lines established from human adrenocortical adenocarcinomas is remarkably short. A computerized search of the National Library of Medicine archives revealed citations of only three putative human cell lines. The SW-13 cell line was established by Leibovitz *et al.* (9) in 1973 from an undifferentiated 'small cell' carcinoma or adrenal cortex. It is not known to secrete any steroid product. In 1977, Fang (10) established a cell line that secreted only estrogen. Our literature search revealed only one further citation to this cell line, by Furuhashi and Fang (11) in 1980. In 1987, Moffett (12) described, in abstract form, an adrenal tumor line that secreted renin and angiotensin. To date, this line has not been described in detail in the medical literature. Thus, none of the three human lines mentioned secrete any of the major steroid products of the normal adrenal cortex and only one, SW-13, has been utilized by multiple investigators. For lack of a suitable human model, many investigators have utilized the Y1 mouse line (13) cultured by Sato and co-workers from a serially transplanted tumor that arose many years previously in a mouse exposed to radiation. The Y1 line has properties unusual in human adrenocortical carcinomas, including adrenocorticotropic hormone-mediated control of steroidogenesis, growth, and morphology.

In this report we describe the establishment and characterization of a permanent functioning culture of a human adrenocortical carcinoma, established and maintained in a defined, serum-free medium. Even after several years in culture, it secretes many steroids characteristic of the glucocorticoid, mineralocorticoid, and androgen pathways of the adrenal cortex.

MATERIALS AND METHODS

Cell Culture Establishment and Characterization

Finely minced tissue, selected from viable nonhemorrhagic portions of a primary adrenocortical carcinoma, were cultured in 96-well micro-

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plates containing growth medium. Four media were utilized, RPMI 1640 or Dulbecco's modified medium supplemented with 10% fetal bovine serum (R10 medium and D10, respectively), RPMI 1640 medium supplemented with hydrocortisone, insulin, transferrin, 17β-estradiol, and sodium selenite (HITES medium), which was originally formulated for the selective culture of small cell lung carcinoma (14, 15), and HITES medium supplemented with 2% fetal bovine serum (H2 medium). Because HITES medium contains two steroids, for steroid secretion studies cells were cultured in RPMI 1640 medium supplemented with sodium selenite, insulin, and transferrin (SIT), with 2% serum (SIT-2) or without serum (SIT-0).

Ultrastructural studies of tumor tissue and cultured cells were performed as previously described (16). For confirmation of tumorigenicity, 5×10^6 cultured cells were inoculated s.c. into athymic nude mice (17). Xenografts so induced were examined by light microscopy after formalin fixation.

Cytogenetic Methods

Slides for cytogenetic studies were prepared by the standard air-dry method from exponentially growing cultures after colcemid treatment (0.1 μg/ml final concentration for 45 min). They were stained with fast G-banding (18) for detailed chromosome analysis and with quinacrine mustard for sex determination by fluorochrome observation. Chromosome number distribution was assessed from 100 metaphases, and the percentage of cells having ploidies higher than the modal chromosome number was estimated by scanning 500 metaphases.

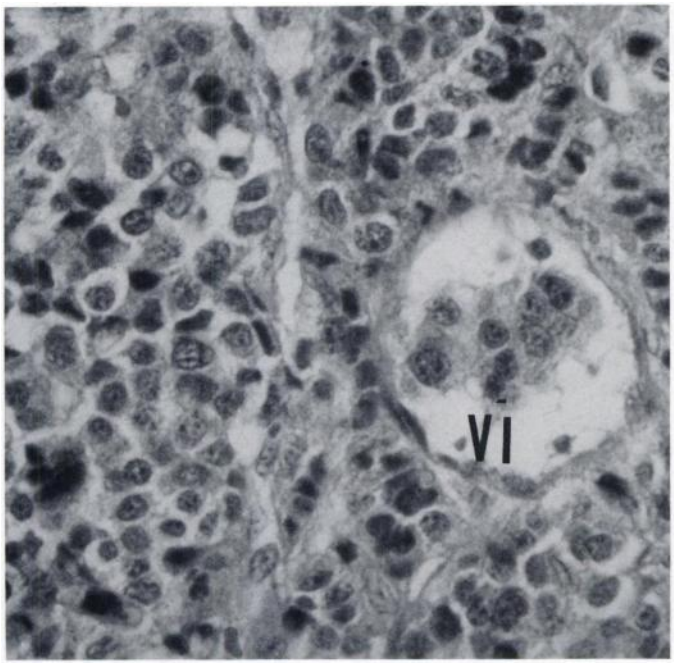

Fig. 1. Adrenocortical tumor from which cell line NCI-H295 was cultured. The cells are relatively small and uniform, although occasional cells are multinucleated and bizarre appearing. Vascular invasion is present (*VI*).

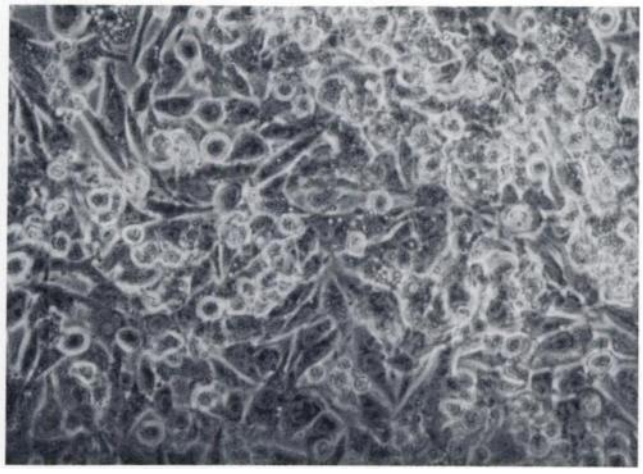

Fig. 2. Phase contrast photomicrograph of NCI-H295 cells cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. The cells are adherent and epithelioid and spindle shaped. In HITES and SIT media, the cells grew as floating cell aggregates lacking substrate attachment.

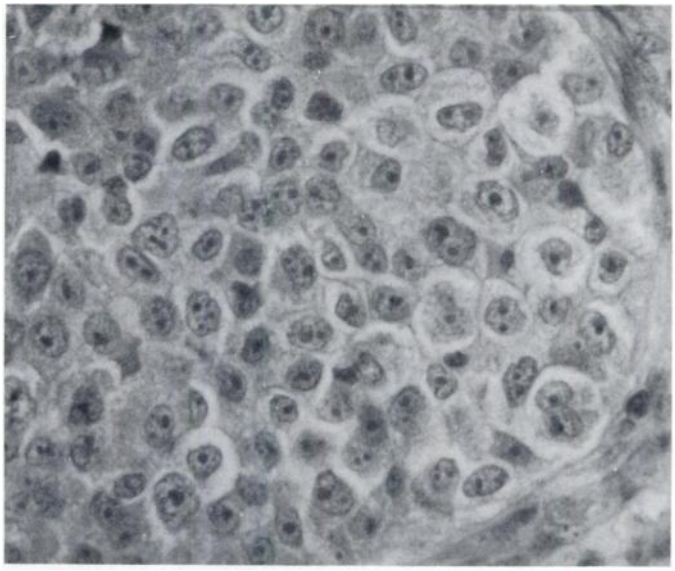

Fig. 3. Histological appearances of xenograft induced by s.c. inoculation of NCI-H295 cells into an athymic nude mouse. The appearances of the xenograft are similar to those of the original adrenocortical carcinoma, although there are fewer multinucleated and bizarre-appearing cells.

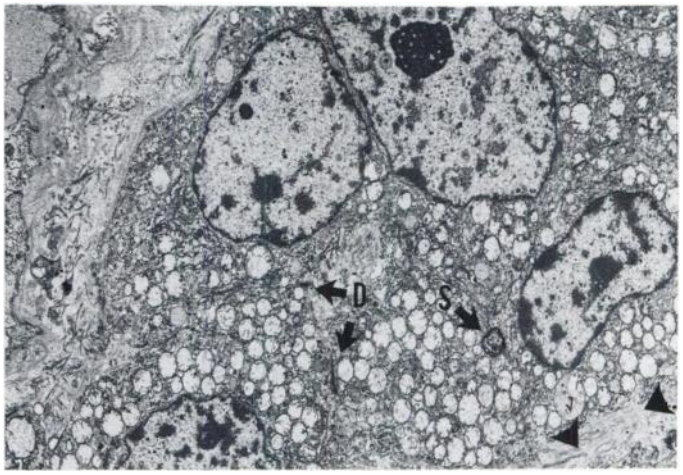

Fig. 4. Ultrastructural appearance of the original tumor. Portions of four nuclei, one with a conspicuous nucleolus, are obvious within epithelioid cells. Large numbers of cytoplasmic mitochondria with cleared (autolyzed) cristae are evident. Less conspicuous is the vesicular endoplasmic reticulum between the mitochondria. A single structure resembling a spironolactone body is present in one cell (*S*), indicative of the steroidogenic character of the tumor cells, especially in context with the above features. Other features include cell to cell attachments typical of epithelia (desmosomes, *D*) and discontinuous basal lamina (*arrowheads*).

Steroid Analyses

Radioimmunoassays. Methodology and validation of steroid analyses using radioimmunoassays after column chromatography have been previously described (19–25). The results were analyzed by logit-log analysis using a computer program.

Mass Spectrometry. Data obtained from RIAs[5] were confirmed and extended by MS analyses, either GC/MS or HPLC/MS. While the

[5] The abbreviations used are: RIA, radioimmunoassay; MS, mass spectrometry; GC, gas chromatography; HPLC, high performance liquid chromatography.

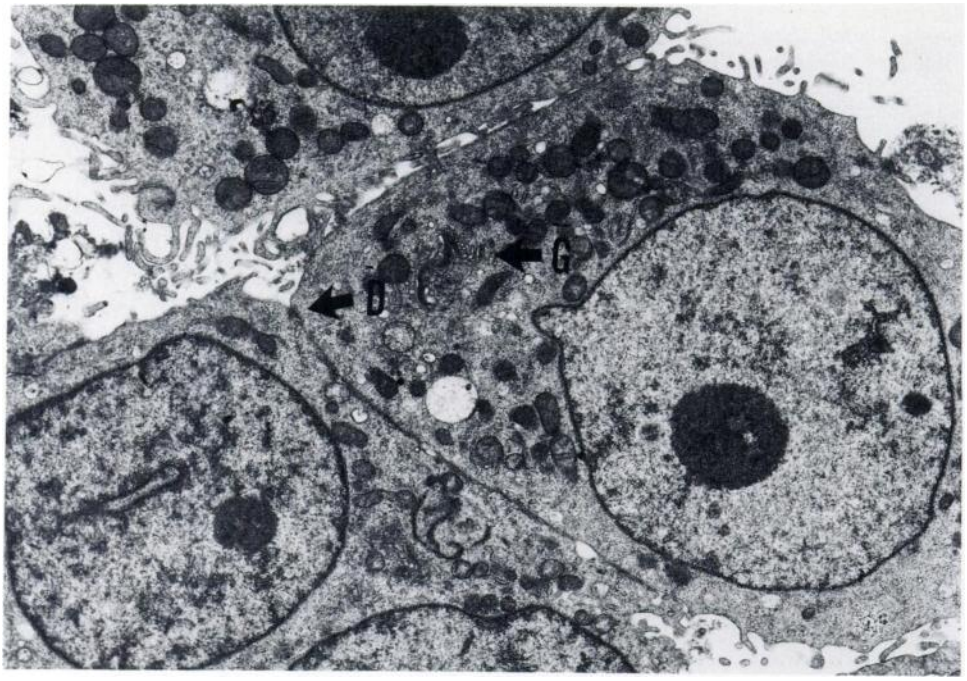

Fig. 5. Ultrastructural appearances of cell line NCI-H295, derived from the tumor described in Fig. 4. The cells remain epithelioid, with desmosomes (*D*), and nucleoli remain prominent. Overall preservation is much better, and the numerous mitochondria are more apparent. Prominent Golgi apparatus is obvious as well (*G*). Features of *in vitro* cultured cells present include absence of basal lamina and numerous microvilli.

separation and identification of steroids by MS has been reviewed by Shackleton (26), because of the complexity and multiple possible permutations of these methods, the ones utilized in the present studies are described in detail. Steroids were extracted from 500-ml samples representing pooled 24-h harvests. Steroids were extracted using Sep-pak cartridges (27) from Waters Associates (Milford, MA). Because the volumes were large, the cartridges were eluted with methanol prior to extraction of further 50-ml aliquots. Following extraction, the methanol eluates were dried under vacuum and reconstituted in 4 ml methanol. To a portion of the steroid extracts was added three internal standards (5α-androstane-3α,17α-diol, stigmasterol, and cholesteryl butyrate), and methyloxime derivatives were made of reactive carbonyl groups. For some steroids, particularly sensitive selected ion-monitoring methods were used, with the crude extract fractionated by Sephadex LH-20 prior to derivatization (28). For GC/MS, samples were separated on a 15-m SPB-1 fused silica capillary column attached to a Hewlett-Packard 5970 mass spectrometer. The samples, dissolved in cyclohexane, were injected splitless with an oven temperature of 50°C. This temperature was held for 3 min and the oven was then rapidly taken up to its starting temperature of 230°C. The temperature was programmed to 310°C at 2°C/min. Mass spectra were obtained by repetitive scanning over the 100–800 atomic mass unit mass range. The total unconjugated steroid production was determined by integrating all the steroid peaks (major and minor, characterized and unknown) and relating this total to the integrated peak areas of the internal standards.

Intact steroid sulfates present in the extract were also analyzed by thermospray HPLC/MS (29). Separation was achieved on a 15-cm C_{18} column housed in a Waters 600 instrument, using the solvent system methanol/0.1 M ammonium acetate (70/30). The eluent for the HPLC passed through a thermospray interface to a VG 30-250 mass spectrometer. The androgen sulfates were detected by negative ion selected ion-monitoring of their molecular anions (29) but were not quantified.

RESULTS

Case History. The patient, a 48-year-old black woman from the Bahamas, was evaluated in October 1980 for weight loss, acne, facial hirsutism, edema, diarrhea, and recent cessation of menses. A computer-assisted tomography scan revealed a large adrenal mass. She was referred to the National Cancer Institute (Bethesda, MD). Her serum cortisol was 11.9 μg/dl, and her 24-h urine excretion levels of cortisol, aldosterone, and 17-ketosteroids were greatly elevated, while her 17-hydroxycorticoids urine levels were near the upper limit of normal (but they failed to suppress with dexamethasone). In December 1980, a 14- x 13- x 11-cm right adrenal mass was removed surgically. The tumor had the histological appearances typical of a malignant adrenocortical carcinoma (30), including abundant eosinophilic cytoplasm and large oval to round nuclei with prominent nucleoli. Many mitoses, pleomorphic cells, and foci of necrosis were present, as well as invasion of perinephric fat and vascular channels, indicative of the highly malignant nature of this tumor (Fig. 1). In February 1981 her tumor recurred and multiple bilateral pulmonary nodules were noted, of which 16 were removed by thoracotomy. She was treated with mitotane and cortisone acetate. In September 1981, further pulmonary nodules and hepatic filling defects were noted. She returned to the Bahamas and died a few months later.

Establishment and Characterization of NCI-H295 Cell Line. A portion of the adrenal tumor was finely minced and placed in culture in microwells with one of four growth media. Initial growth, at a slow rate, occurred in all four media. In R10 and D10 media, fibroblast overgrowth of the tumor cells eventually occurred, and the cultures were discarded. In HITES medium, 'pure' tumor cell growth as floating aggregates occurred. In H2 medium, tumor cells grew as both floating and adherent colonies. However, because a few adherent fibroblasts also were present, the floating cells were selected for passage. After about 1 year, the HITES culture was determined to be the most vigorous and was the only one selected for continuous cell propagation. After establishment, the cells could readily be adapted to culture in R10 medium. In HITES medium, (which is devoid of attachment factors), the cells grow as floating aggregates of loosely aggregated cells, while in R10 medium they grow as attached epithelioid or spindle-shaped cells (Fig. 2). Most of the characterization studies described herein were performed in HITES medium.

Five of six athymic nude mice inoculated s.c. with 5×10^6 NCI-H295 cells developed tumors at the inoculation site 6 to 9 weeks later. The xenografts so induced had histological appearances similar to the adrenocortical carcinoma present in the patient (Fig. 3), although bizarre, multinucleated cells were more common in the tumor.

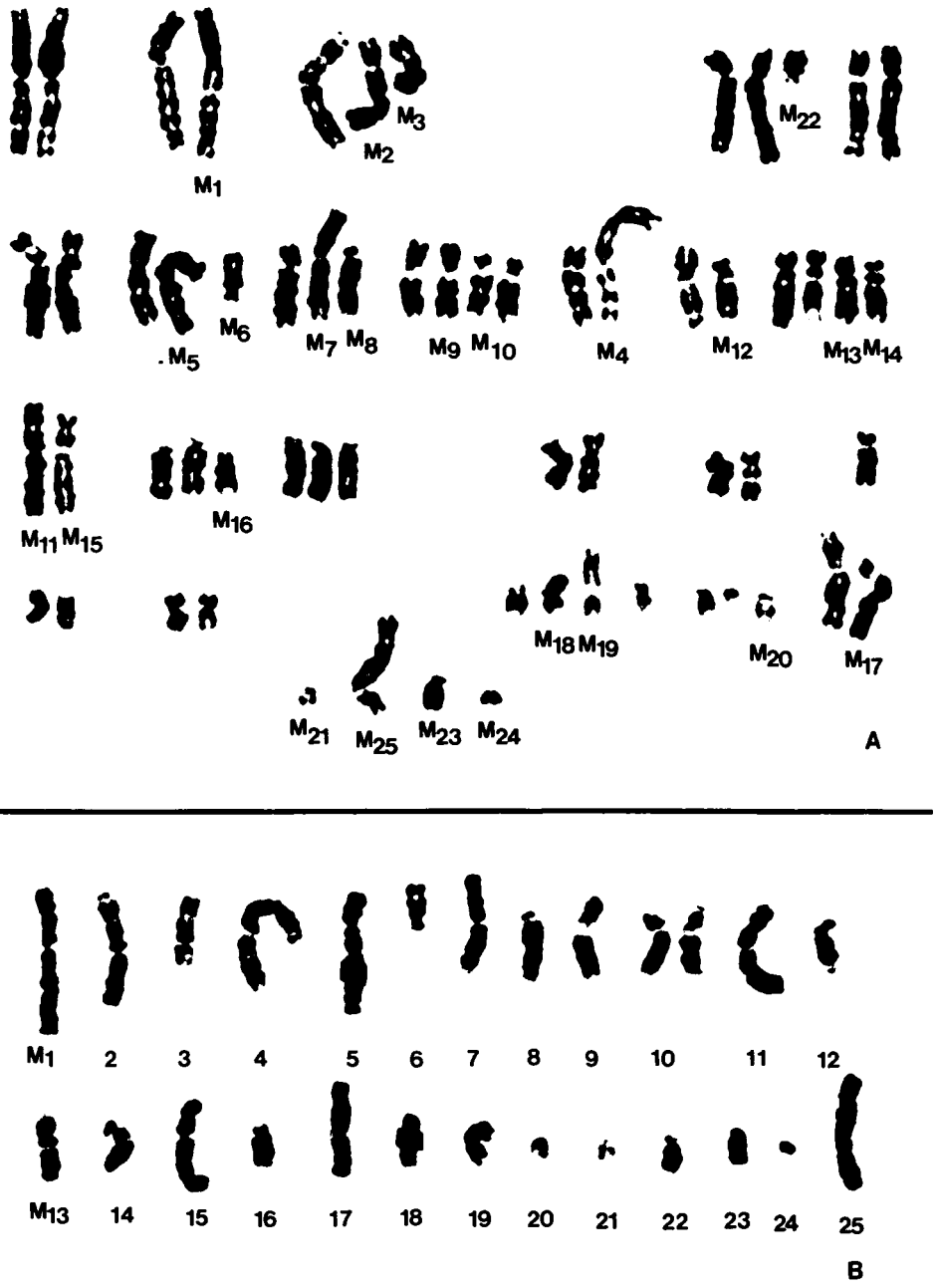

Fig. 6. *A*, trypsin G-banded karyotype from the major subline of NCI-H295. The 25 common marker chromosomes are numbered. Marker chromosome 12 is deletion 11p. *B*, set of marker chromosomes from another cell. They are identified by the number preceded by the letter *M* on the *extreme left* in each *row*.

Ultrastructural Studies. Electron microscopic studies of the tumor and the cell line established from it confirmed the steroidogenic character of both (2, 31). The original tumor cells (Fig. 4), although suffering moderate autolytic damage, were characterized by enormous numbers of mitochondria (with artifactually lysed cristae), vesicular smooth endoplasmic reticulum, epithelioid nuclei with prominent nucleoli, and even rare cytoplasmic structures resembling spironolactone bodies, found only in adrenal cortical cells. Epithelial cell to cell attachments were noted. Basal lamina also were present, although widely disrupted, a feature more characteristic of carcinoma as opposed to adenoma cells.

The cultured tumor cells, even from late passages, clearly retained many of the same features noted above (Fig. 5). In particular, the nuclei remained clearly epithelioid, with single prominent nucleoli; the cytoplasm contained large numbers of mitochondria (without autolytic changes), moderate vesicular smooth endoplasmic reticulum, and prominent Golgi apparatus, features typical of secretory activity. The latter feature was less conspicuous in the original cells, perhaps masked by the widespread autolytic changes noted above. Cell to cell attachments, like in the original tumor, remained conspicuous. Basal lamina (a feature usually lacking in cultured cells) were no longer present. Microvilli, a feature characteristic of cultured epitheloid cells, were quite prominent.

Cytogenetic Studies. Cytogenetic examination of NCI-H295 revealed a hypertriploid human cell line with the modal chromosome number, 62, occurring in 30 of 100 cells counted. The distribution of chromosome numbers was as follows (number of chromosomes, number of cells): 55, 3; 56, 2; 57, 5; 58, 4; 59, 12; 60, 17; 61, 15; 62, 30; 63, 10; 64, 2. The rate of higher ploidies was 3.8%. No Y or Y-like chromosomes were found by fluorescent microscopy.

Karyotypic analysis of 38 cells demonstrated a total of 65 marker chromosomes. Of these, 25 were common to most cells (Fig. 6). Two subline-specific markers occurred in some cells (Fig. 6*B*), and the remaining 38 were detected only once. Most of the break-union sites occurred pericentrically, although one was noted at 7q36.

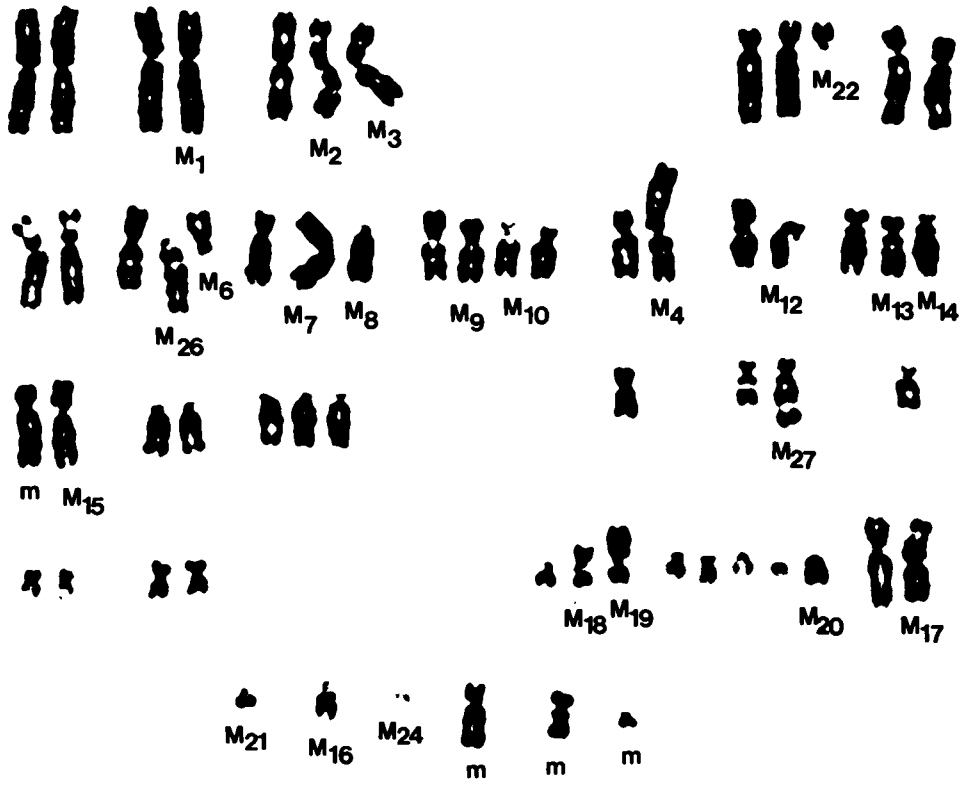

Fig. 7. Trypsin G-banded karyotype of the minor subline, showing its unique chromosome pattern, *i.e.*, the presence of del(M5)(p11) (M26) and der(17) t(17;?)(q25;?) (M27) and the single copy of M17. Note also the lack of M5 of the major subline (Fig. 6). Otherwise, both sublines have nearly identical chromosome composition. The other common markers are identified by the number corresponding to that shown in Fig. 6*A*.

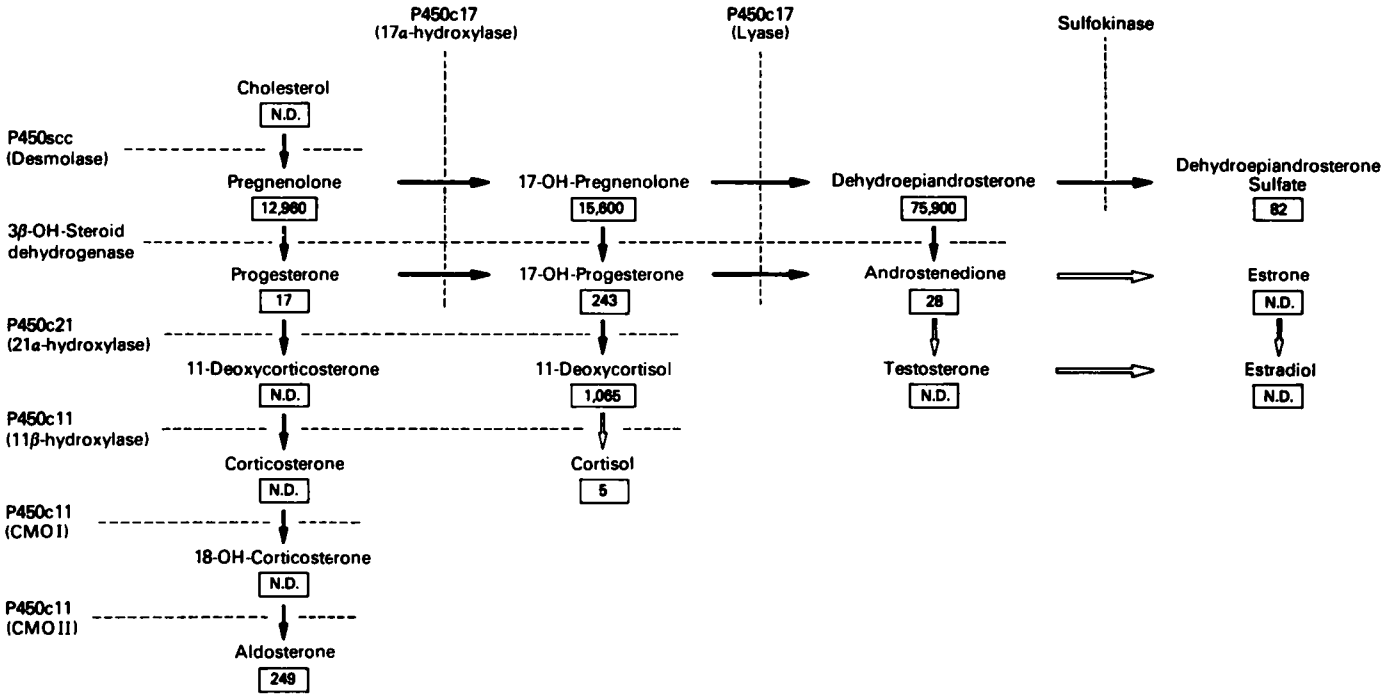

Fig. 8. Steroid selection by NCI-H295 cells cultured in serum-supplemented SIT medium (SIT-2), as determined by RIA. The major pathways of adrenal cortical steroidogenesis are outlined. The *open arrows* indicate reactions that occur predominantly in peripheral tissues. The enzymes involved are indicated in the *left* and *upper columns*, and the corresponding reaction or reactions are indicated by *dashes*. The *boxed figures* contain the values (in ng/10^8 cells/24 h) of the steroid products mentioned above present in the supernatant fluids of the cultured cells (mean values of two experiments performed with cells grown in HITES medium). *N.D.*, not done.

A minor subline occurred in about 16% of cells. This subline possessed, instead of der(7)t(7;7)(q36;p13) (or M5) and the paired N17 seen in karyotypes of the major subline, two new markers, del(M5)(7p11) (or M26) and der(17)t(17;?)(q25;?) (or M27), and the single N17 (Fig. 7). All other chromosomes were otherwise identical. Neither double minutes nor heterogeneously staining regions were detected. Normal N13 was absent. N15 and N22 constantly had three and four copies/cell, respectively. Other normal chromosomes (either one or two copies/metaphase) were present in over 90% of cells.

Steroid Secretion. Steroid studies were performed after the cells had been in culture for 7–10 years. RIA analyses of clarified supernatant fluids of the cells cultured in SIT medium (with or without 2% bovine serum) contained varying concen-

Table 1 *Major unconjugated steroids present in supernatant fluids of NCI-H295 cells cultured in serum-containing and serum-free media, as identified by GC/MS*

Of more than 30 steroids found by GC/MS in serum-containing medium, the relative percentages of the 10 most abundant are listed in the table. The peak numbers correspond to the peaks illustrated in Fig. 9. In addition to the above listed steroids, the following were identified, none of them representing more than 3% of the total: androsterone, epiandrosterone, testosterone, 17α-hydroxyprogesterone, estrone, 16α-hydroxydeoxyandrostenedione, 19-hydroxyandrostenedione, 16α-hydroxypregnenolone, corticosterone, 18-hydroxycortisol, estradiol, and 5-androstene-3β,16α,17β-triol. Several other steroids remain unidentified including at least two hydroxyandrostenediones and an important X,19-dihydroxyandrostenedione. The sulfated forms of androgens identified by HPLC/MS (see text) are not included in this table.

Steroid	% of total SIT-2	% of total SIT-0
Cortisol (peak 10)	21.3	0.6
11β-Hydroxyandrostenedione (peak 6)	15.9	4.6
Androstenedione (peak 3)	11.7	6.8
5-Pregnene-3β,17α,20α-triol (peak 8)	5.1	3.6
Dehydroepiandrosterone (peak 2)	4.5	11.0
Pregnenolone (peak 5)	3.4	3.5
5-Androstene-3(α),16α,17β-triol (peak 4)	3.4	3.5
17α-Hydroxypregnenolone (peak 7)	3.3	4.8
3α-Hydroxy-5-androsten-17-one (peak 1)	3.0	1.8
11-Deoxycortisol (compound S) (peak 9)	2.6	<1.5
Total steroid secretion (μg/10⁶ cells/24 h)	2.83	0.72

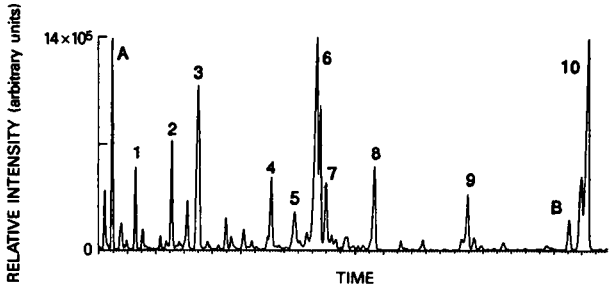

Fig. 9. GC/MS total ion current chromatogram of methyloxime-trimethylsilyl esters of steroids formed in media containing fetal bovine serum. The 10 principal components are listed in Table 1. For certain compounds, *e.g.*, 11β-hydroxyandrostenedione (peak 6) and cortisol (peak 10), two peaks have been assigned, which represent the *syn*- and *anti*-forms of the methyloxime derivatives. Peaks A and B are internal standards.

trations of several steroids characteristic of adrenocortical cells (Fig. 8). In particular, very high concentrations of pregnenolone, 17-hydroxypregnenolone, and dehydroepiandrosterone were present, along with more modest concentrations of 17-hydroxyprogesterone, aldosterone, 11-deoxycortisol, progesterone, androstenedione, and dehydroepiandrosterone sulfate. Cortisol concentrations were below those that could be detected with confidence. Unused culture media and spent media of control lung adenocarcinoma cells (grown in HITES medium) lacked detectable levels of these steroids. Dehydroepiandrosterone and 17-hydroxyprogesterone were not detected in cell pellet homogenates of NCI-H295 cells.

GC/MS assays indicated that total unconjugated steroid secretion in serum-supplemented medium (SIT-2) was 2.83 μg/10⁶ cells/24 h and about 4-fold less in serum-free medium. More than 30 steroids were detected in supernatant fluids of cells cultured in SIT-2, of which about 20 were identified. The 10 major unconjugated steroids, listed in Table 1, accounted for about 70% of the total, and the separation of these is illustrated in Fig. 9. Representative mass spectra of methyloxime-trimethylsilyl ether derivatives are illustrated in Fig. 10. These and all other mass spectra were identical to those of equivalent reference compounds, and the peaks had the same retention times. No steroids were identified in control samples of the two media. The presence of the sulfated steroids dehydroepiandrosterone sulfate, epiandrosterone sulfate, and androsterone sulfate was confirmed by thermospray HPLC/MS (Fig. 11). Certain expected steroids were not identified by MS, including progesterone, deoxycorticosterone, 18-hydroxycorticosterone, and aldosterone. However, the finding of 18-hydroxycortisol confirmed that 18-hydroxylation of C_{21} steroids occurred. Particularly sensitive selected ion-monitoring methods were used to no avail in the search for these compounds.

Because serum-supplemented media contain cholesterol and a mixture of steroids, the studies also were performed in serum free SIT medium (Table 1). Cell growth in SIT-0 medium was considerably slower than in SIT-2 medium. The population doubling time of the cells was considerably longer in SIT-0 than in SIT-2 medium. The total concentration of secreted steroids was decreased about 4-fold in SIT-0 medium (Table 1), and C_{21} steroid formations was greatly decreased relative to C_{19} steroid formation (Table 1). RIA assays confirmed that a considerable decrease in steroid secretion occurred in SIT-0 (data not shown).

DISCUSSION

In many ways the patient from whom cell line NCI-H295 was established had the typical clinical, biochemical, and pathological profile of adrenocortical carcinoma (3, 32). She was 48 years old, presented with a large locally invasive malignant tumor, which later metastasized to the lungs and liver, and had clinical and biochemical evidence of excessive secretion of glucocorticoids and ketosteroids. However, she also had hyperaldosteronism, a relatively rare finding in association with adrenocortical carcinoma.

Our ability to establish in continuous culture a functioning adrenocortical carcinoma, when many others have failed, may reflect our use of a fully defined medium (HITES) originally formulated for the growth of small cell carcinoma of the lung (14, 15). Initial tumor cell growth in conventional serum-containing medium was unsuccessful due to fibroblast overgrowth, although the culture could be adapted to growth in such media once it became established in serum-free medium. Initial or continued growth of NCI-H295 cells did not require fibroblast growth factor, a potent stimulator of normal and cultured adrenocortical cells (33, 34). Short term culture of bovine adrenocortical cells can be achieved in a fully defined medium supplemented with selenium, insulin, transferrin, lipoproteins, albumin, fibroblast growth factor, and vitamins (34). NCI-H295 cells could be adapted to growth in a simple medium supplemented only with selenium, insulin, and transferrin. These findings suggest that NCI-H295 cells may secrete autocrine growth factors. Of interest, SW-13 cells are reported to secrete as yet unidentified autocrine growth factors (35).

The ultrastructural features of steroid-secreting cells include the characteristic appearance of mitochondrial cristae (lamellar or tubulovesicular) and the presence of a well developed smooth endoplasmic reticulum (2, 31). These features reflect the subcellular location of the enzymes involved in steroidogenesis, and they were present in both the tumor and cultured cells. Lipid droplets are a prominent feature of adrenocortical cells, especially in species in which most of the cholesterol is derived from lipoproteins (2). Lipid droplets were inconspicuous in NCI-H295 cells, perhaps reflecting the entirely endogenous production of cholesterol requirements (see below).

Cytogenetic studies demonstrated a highly aneuploid line containing multiple marker chromosomes, 25 of which were common to most cells. However, karyotypes of individual cells were similar. We found only two reports of cytogenetic studies

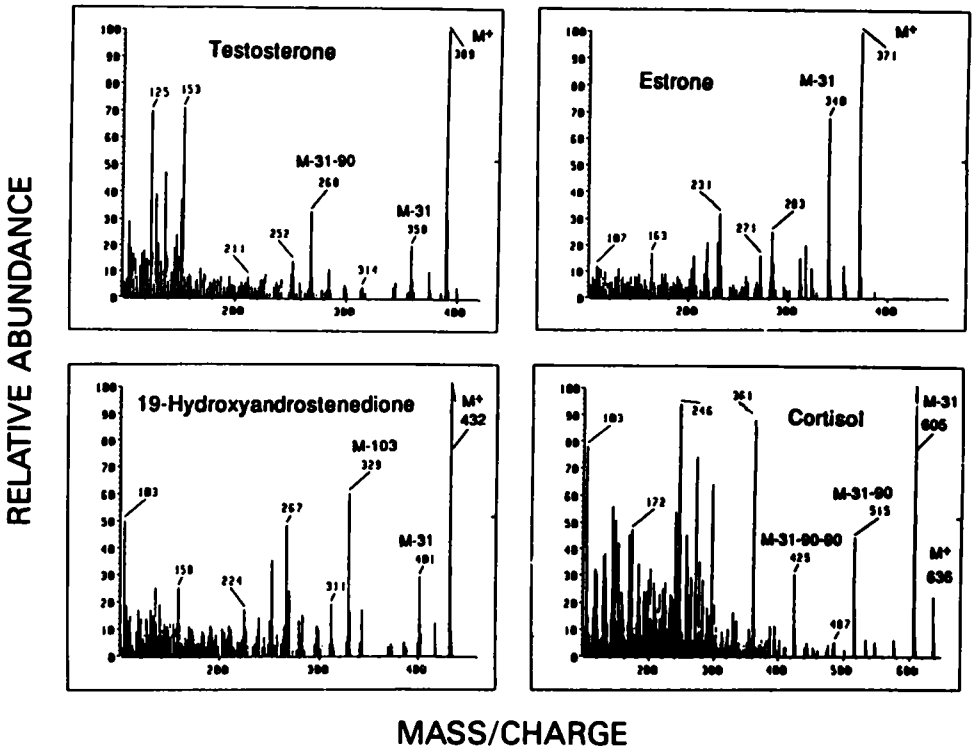

Fig. 10. Representative mass spectra of methyloxime-trimethylsilyl esters of isolated steroids. These spectra (and all others) were identical to those of authentic samples and the peaks had equivalent retention times. The molecular ions are represented by M^+ and in many compounds major fragmentations are caused by loss of the oxime (−31) and trimethylsilanol (−90) moieties.

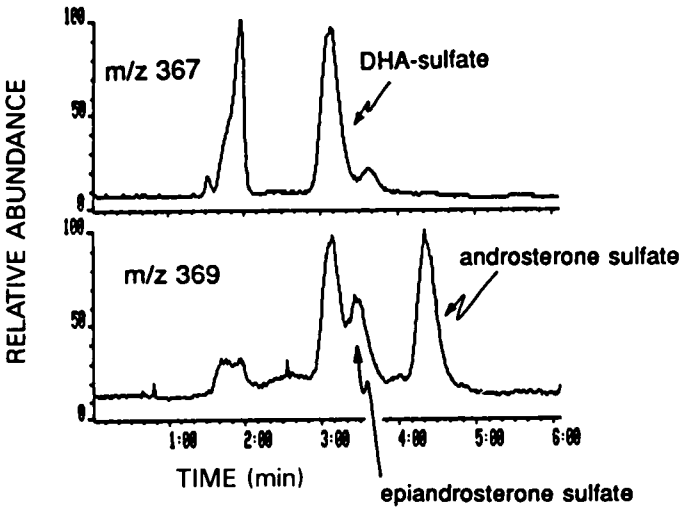

Fig. 11. Thermospray HPLC/MS separation of intact androgen sulfates. The chromatograms are selected ion recordings of the molecular anions of DHA-sulfate (m/z 367) and the saturated androgen sulfates (m/z 369). The retention times and mass spectra were identical to appropriate reference compounds. The peaks in the m/z 369 recording are in reality about 1/5 the size of the peaks in the m/z 367 recording. The peaks that were identified are labeled. The early peak in the m/z 367 recording could possibly be the sulfate of the 3α-epimer of DHA or testosterone.

on human adrenocortical tumors. In a functioning carcinoma, Limon *et al.* (36) found abnormalities involving only chromosomes 4 and 11. However, in a nonfunctioning carcinoma, they found several marker chromosomes (37). In addition, we have performed cytogenetic studies of the nonfunctioning human adrenocortical carcinoma cell line SW-13.[6]

Despite the relative paucity of cytogenetic studies in human adrenocortical carcinomas and cell lines, certain common features are noted. Chromosomes 1, 7, 9, 14, 16, and 20 had contributed to the formation of marker chromosomes in three of the four adrenocortical carcinomas examined. Among the marker chromosomes in these three carcinomas, N7 had the highest incidence, involving 14 markers; N14 and N20 involved 8 markers, and N9 and N12 involved 7 markers. While most of the break union-sites, including those involving N7, occurred pericentrically, 7q36 was a break-union site noted in two different carcinomas (NCI-H295 and the nonfunctioning carcinoma) (37). Of interest, frequent structural abnormalities of N7 also are characteristic of cell lines derived from colorectal carcinomas, melanomas, and gliomas (38–40), and 7q36 is a frequent break-union site in colon carcinomas.[6]

The major function of the adrenal cortex is to produce biologically active steroids (some of which are converted to their final products in other organs). The cortex produces more than 40 different steroids (2, 41). Both glucocorticoids and mineralocorticoids have 21 carbon atoms and are uniquely produced by the cortex. The cortex is also a major source of C_{19} androgens and a minor source of C_{18} estrogens and progestins. The initial and final steps in steroidogenesis occur in the mitochondria and the intermediate ones in the smooth endoplasmic reticulum. The major events in steroidogenesis will be briefly outlined below and in Fig. 8 and related to steroid secretion by NCI-H295 cells.

All of the adrenal steroids are derived from cholesterol. The major source of adrenal cholesterol (about 80%) in humans is plasma cholesterol esters derived from circulating lipoproteins (2, 6, 7). In addition, endogenous formation occurs from acetate. Presumably NCI-H295 cells are capable of synthesizing all of the cholesterol required for steroidogenesis, because steroid synthesis occurred in fully defined, serum-free, cholesterol-free medium. Conversion of cholesterol to pregnenolone in the mitochondria is the rate-limiting step in normal adrenal steroid biosynthesis. The side chain cleavage desmolase complex, consisting of a reaction-specific cytochrome P_{450}, cleaves the side chain from the 27-carbon cholesterol molecule to form a 21-carbon molecule that functions as the steroid skeleton. The

[6] T. R. Chen, unpublished data.

relatively high concentrations of pregnenolone secreted by NCI-H295 cells suggest that it is formed at a faster rate than it can be metabolized.

Pregnenolone is transported to the smooth endoplasmic reticulum, where it is converted to progesterone by 3β-hydroxysteroid dehydrogenase-isomerase complex. Cortisol synthesis proceeds via 17α-hydroxylation of pregnenolone to form 17α-hydroxyprogesterone, which, in turn, is converted by 21-hydroxylation to 11-deoxycortisol. This compound is further hydroxylated in the mitochondria by 11β-hydroxylase to cortisol, the principal glucocorticoid. Glucocorticoid formation occurs almost exclusively in the zona fasciculata. Aldosterone, the principal mineralocorticoid, is also formed from progesterone, in the zona glomerulosa, by sequential 21- and 11β-hydroxylation via corticosterone and 18-hydroxycorticosterone. The production of adrenal androgens requires prior 17α-hydroxylation and thus does not occur in the zona glomerulosa. The major quantitative production of androgens is by conversion of 17α-hydroxypregnenolone to the C_{19} steroids dehydroepiandrosterone and its sulfated conjugate. The other major adrenal androgen, androstenedione, is produced from 17α-hydroxyprogesterone. It can be converted (mainly in other tissues) to testosterone as well as to estrogens. NCI-H295 cells secrete relatively large quantities of the major adrenal androgens and smaller amounts of testosterone and estrogen.

Our findings suggest that a major pathway of pregnenolone metabolism in NCI-H295 cells is androgen formation, although mineralocorticoids and glucocorticoids are also synthesized. In many adrenocortical carcinomas, secretion of the end products of the various steroid pathways (cortisol, aldosterone, androstenedione, and dehydroepiandrosterone sulfate) is modest, and 11β-hydroxylase activity is decreased or absent (30). Cortisol and corticosterone formation was demonstrated in NCI-H295 cells by GC/MS and aldosterone formation by RIA. While the presence of aldosterone was not demonstrated by GC/MS, the presence of 18-hydroxylation was confirmed by the presence of 18-hydroxycortisol. The 18-hydroxylated cortisols are relatively recently described adrenal steroids excreted in exaggerated quantities in patients with primary aldosteronism (42). Our findings indicate the presence in NCI-H295 cells of all of the major adrenocortical enzyme systems, including 11β-hydroxylase, desmolase, 21α-hydroxylase, 17α-hydroxylase, 18-hydroxylase, lyase, and sulfokinase. In addition, formation of estrogens (which are not prominent products of the adrenal cortex) indicated the presence of aromatase. The differences in the formation of the C_{21} end products by the two assay methods (performed more than 1 year apart) suggest shunting between mineralocorticoid and glucocorticoid pathways. Of interest, the two assay methods revealed very similar results for secretion of dehydroepiandrosterone in SIT-2 medium. In serum-free medium, androgens constituted almost all of the steroids detected by both assays, confirming that shunting between pathways could occur.

Steroid storage granules have not been unequivocally demonstrated in adrenocortical cells, and the precise mechanism of hormone release is controversial. The most widely accepted theory for hormone release is that they freely diffuse throughout the cytosol and plasma membrane (2). However, a more complex mechanism involving active transport has also been postulated (2, 43). Our failure to demonstrate steroid storage in NCI-H295 cells in the face of considerable extracellular concentrations is in favor of the freely diffusible theory.

The NCI-H295 cell line continues to secrete multiple steroids even after many years in culture and when grown in a fully defined medium devoid of steroids, serum, and fibroblast growth factor. It should prove of value in studying the regulation, metabolic pathways, and enzymes involved in steroid formation and secretion. In addition, it may provide insights into the biology and treatment of adrenocortical carcinoma.

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