Proc. Natl. Acad. Sci. USA
Vol. 78, No. 2, pp. 1028–1032, February 1981
Cell Biology

Evidence for a higher molecular weight precursor of cholesterol side-chain-cleavage cytochrome *P*-450 and induction of mitochondrial and cytosolic proteins by corticotropin in adult bovine adrenal cells

(cell culture/cortisol production/protein radiolabeling/*in vitro* translation)

RAYMOND N. DUBOIS*, EVAN R. SIMPSON*†‡, JANETTE TUCKEY†‡, J. DAVID LAMBETH§, AND MICHAEL R. WATERMAN*¶

Departments of *Biochemistry and †Obstetrics and Gynecology, and ‡The Cecil H. and Ida Green Center for Reproductive Biology Sciences, The University of Texas Health Science Center at Dallas, Dallas, Texas 75235; and §Department of Biochemistry, Emory University School of Medicine, Atlanta, Georgia 30322

Communicated by Ronald W. Estabrook, October 29, 1980

ABSTRACT **Adult bovine adrenal cortical cells in monolayer culture were used to study the induction of cholesterol side-chain-cleavage cytochrome *P*-450 by corticotropin (ACTH). In the presence of 1 μM ACTH, there was a 4-fold increase in cortisol production by these cells over a 72-hr period and a corresponding increase in total cytochrome *P*-450 content. The incorporation of [^{35}S]methionine into a number of cellular proteins was stimulated by the presence of ACTH in the culture medium, whereas the incorporation into other proteins was decreased. The temporal profile of these changes varied from one protein to another. Examination of the incorporation of [^{35}S]methionine into mitochondrial protein showed an increased production of a radiolabeled protein that comigrated with the form of cytochrome *P*-450 known as side-chain-cleavage cytochrome upon incubation with ACTH. Thus, it appears that the cytochrome *P*-450$_{scc}$ content is increased in bovine adrenal cortical cells exposed to ACTH. Cytochrome *P*-450$_{scc}$, synthesized in a cell-free translation system directed by RNA isolated from bovine adrenal cortical tissue or from cells, had a molecular weight of 54,500. Cytochrome *P*-450$_{scc}$ isolated from bovine adrenal mitochondria had a molecular weight of 49,000. Thus, cytochrome *P*-450$_{scc}$ is synthesized as a larger precursor that must be processed by proteolytic cleavage before or upon insertion into the mitochondrion.**

The molecular events by which corticotropin (ACTH) stimulates cholesterol side-chain cleavage by adrenal mitochondria are being examined by using cultures of adult bovine adrenal cortical (BAC) cells. This cell culture system has been described and characterized (1–3). These cells are responsive to ACTH, as shown by increased cortisol output during the first few days of culture (3, 4). The rate-limiting step in cortisol biosynthesis is the first step in the pathway of cholesterol metabolism to steroid hormones—i.e., cholesterol side-chain cleavage (5). This reaction is catalyzed by a form of cytochrome *P*-450 known as side-chain-cleavage cytochrome *P*-450 (*P*-450$_{scc}$), which is located in the inner membrane of the mitochondrion (6–9). Two other mitochondrial proteins, adrenodoxin and adrenodoxin reductase, serve to transfer electrons from NADPH to *P*-450$_{scc}$ and are therefore also required for the conversion of cholesterol to pregnenolone (10, 11).

ACTH is known to effect both an acute and a long-term (chronic) stimulation of the cholesterol side-chain-cleavage reaction. Our study was designed to characterize the long-term stimulation of cholesterol side-chain cleavage by ACTH. We examined radiolabeling profiles of proteins in BAC cells maintained in monolayer culture at various times after addition of ACTH to the culture medium and found a complex pattern of changes. Examination of radiolabeled proteins in mitochondria isolated from such cells suggests that the level of *P*-450$_{scc}$ is increased by ACTH administration. By using an antibody directed toward bovine adrenal cytochrome *P*-450$_{scc}$, we show that *P*-450$_{scc}$ is synthesized *in vitro* as a larger molecular weight precursor that must be processed before or on insertion into the inner mitochondrial membrane.

MATERIALS AND METHODS

Cell Preparation and Culture. The procedure used for bovine adrenal cell preparation and culture was essentially that described by Gospodarowicz *et al.* (1). Bovine adrenal glands were obtained from freshly slaughtered steers and transported to the laboratory on ice. Scrapings of the fasciculata zone were prepared under sterile conditions; placed in Hanks' balanced salt solution containing 25 mM Hepes buffer (pH 7.2) (GIBCO), collagenase-dispase at 2.0 mg/ml (Boehringer Mannheim), and DNase at 0.2 mg/ml (Boehringer Mannheim); and shaken for 30–45 min at 37°C. After the mixture was centrifuged at 1000 rpm for 5 min, the supernatant was removed and the digestion was repeated. The cells were pelleted by centrifugation, washed twice with 25 mM Hepes buffer in Hanks' solution, and plated in 60- or 100-mm culture dishes (Falcon Plastics, Cockeysville, MD) at a density of approximately 10^6 cells per 25 cm^2 in Ham's F-12 medium containing 12.5% horse serum, 2.5% fetal calf serum, 1% antibiotic antimycotic solution, 1% kanamycin, 0.5% mycostatin, and fibroblast growth factor at 50 ng/ml (kindly provided by Denis Gospodarowicz). The medium was replaced every 2 or 3 days, and generally, by 6 days, confluency was attained. At this time, the medium was exchanged for growth factor-, antibiotic-free F-12 medium for an additional 24 hr. Thereafter, cells were incubated in growth factor-, antibiotic-free F-12 medium in the presence or absence of 1 μM ACTH$_{(1-24)}$ (Cortrosyn, Organon) for periods up to 72 hr. The medium was replaced daily, and samples were stored frozen until assayed for cortisol by radioimmunoassay (12).

General Procedures. The *P*-450 content was quantified in sonicates of adrenal cells by measuring the difference between the (reduced CO-cytochrome − reduced *P*-450) difference spectra at 450 and 490 nm (13) with an Aminco DW2 spectro-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: ACTH, corticotropin (adrenocorticotropic hormone); BAC, bovine adrenal cortical; *P*-450$_{scc}$, cholesterol side-chain-cleavage cytochrome *P*-450.

¶To whom reprint requests should be addressed.

photometer (American Instruments, Silver Spring, MD). The sonicates were prepared by removing the medium from the culture dishes (100 mm), washing the cells twice with 5 ml of Gey's balanced salt solution, and scraping the cells from the dishes into 5 ml of Gey's solution by using a rubber policeman. After centrifugation and resuspension in 5 ml of the same solution (three times), the cell pellet was suspended in 1 ml of 0.1 M phosphate buffer (pH 7.5), and the suspension was subjected to ultrasonic disintegration for 10 sec in a Branson model W185D sonifier (Plainview, NY) equipped with a microtip. Finally, the sonicate was homogenized by hand, using a Potter–Elvehjem homogenizer, just before spectrophotometric examination. All manipulations were performed at 4°C.

$NaDodSO_4$/polyacrylamide gel electrophoresis was conducted as described by Laemmli (14). Cellular protein concentration was determined according to the method of Kuno and Kihara (15).

Radiolabeling of BAC Proteins. To examine changes in cellular protein synthesis in response to ACTH stimulation, BAC cells (60-mm culture dishes) were radiolabeled for 30 min with [^{35}S]methionine (120 μCi/2 ml of culture medium; 1 Ci = 3.7×10^{10} becquerels) prior to harvesting. After incubation, the medium was removed and the dishes were washed three times with Gey's balanced salt solution. The cells were treated with 1 ml of lysing buffer (0.0625 M Tris, pH 6.8/2% $NaDodSO_4$/10% glycerol/5% 2-mercaptoethanol) and then transferred to a conical centrifuge tube, and the mixture was heated for 2 min in a boiling water bath. The lysate was lyophilized and stored at −70°C before electrophoresis.

Mitochondria were isolated from washed cells that were radiolabeled as described above by freeze-thawing in 20 mM 4-morpholinepropanesulfonic acid (Mops) buffer, pH 7.4/0.5 mM EDTA/250 mM sucrose. Cell debris and nuclei were removed by centrifugation at 200 × *g* for 10 min, and the supernatant fraction was centrifuged at 13,000 × *g* for 10 min to pellet the mitochondria. This pellet was washed by resuspension in isolation buffer, centrifuged, and subjected to electrophoresis.

Cell-Free Translation of Cellular RNA. RNA for *in vitro* cell-free translation was isolated from two sources. In one instance, freshly removed adrenal glands were partially dissected at the slaughterhouse. The dissected cortical tissue was frozen immediately in liquid nitrogen for transport to the laboratory. Total RNA was isolated from this tissue by the guanidine hydrochloride extraction procedure (16). In the second instance, RNA was isolated from BAC cells maintained in monolayer culture (100-mm dishes) by the phenol/chloroform extraction procedure (17).

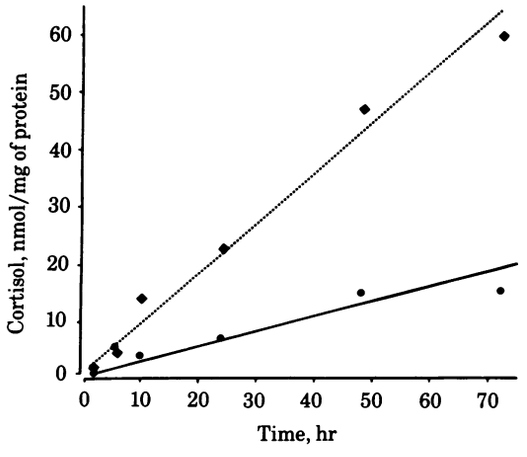

FIG. 1. Time course of cortisol production in monolayer cultures of BAC cells in the presence (◆) or absence (●) of ACTH. Cumulative cortisol values from duplicate culture dishes are given at each time point.

The RNA obtained from both sources was translated in a commercially available rabbit reticulocyte translation system (18) (New England Nuclear) using [^{35}S]methionine as the radiolabel. In all cases, RNA samples were titrated in the translation system to ensure that the amount used in the experiments lay on the linear portion of the radioactivity incorporation curve. Newly synthesized $P\text{-}450_{scc}$ was isolated from the total translation products by the procedure of Ivarie and Jones (19) using Pansorbin (Calbiochem) as the source of protein A. Antibody to $P\text{-}450_{scc}$ was obtained by injection of purified protein into the popliteal lymph nodes of rabbits (20), followed by subcutaneous booster injections. The IgG fraction from serum was prepared by precipitation with 33% ammonium sulfate. Purified bovine adrenal cytochrome $P\text{-}450_{scc}$ was isolated according to the method of Seybert *et al.* (21).

RESULTS

Cortisol Production and Cytochrome *P*-450 Content. The response of BAC cells in monolayer culture to 1 μM ACTH included a 4-fold increase in the output of cortisol. Time courses of cortisol production during a 72-hr period by cells treated with ACTH and by cells maintained in the absence of ACTH are shown in Fig. 1. In the absence of ACTH, the rate of cortisol production was low, but ≈6 hr after ACTH treatment, the rate of cortisol production was greater than control rates and, within 24 hr, cortisol secretion was 4-fold greater in ACTH-treated than in nontreated cells.

The *P*-450 content was estimated spectrophotometrically to be 85 pmol/mg of cellular protein in untreated cells and 443 pmol/mg of cellular protein after 72 hr of ACTH treatment. This 5-fold increase should be considered an estimate in view of the spectral overlap between *P*-450 and cytochrome oxidase. However, at all time points examined (24 hr and beyond) after ACTH addition to the culture medium, there was an increased level of *P*-450 compared with that in cells maintained in the absence of ACTH. The amount of *P*-450 continued to increase at least up to 72 hr after ACTH addition.

Radiolabeling of Cellular and Mitochondrial Proteins. Examination of the pattern of radiolabeling of proteins in the BAC cells showed that ACTH treatment had a dramatic effect. Autoradiographs of the profiles obtained after electrophoresis of the radiolabeled proteins derived from cells incubated for various periods of time in the presence or absence of ACTH are shown in Fig. 2, and the extent of radiolabeling of cellular protein, which was quite constant after the first few hours of ACTH treatment, is given in Table 1. ACTH increased the incorporation of radiolabel into several protein bands and decreased the incorporation into several others. Densitometric scans of several of the lanes from the autoradiograph shown in Fig. 2 are shown in Fig. 3. The densities of the bands at M_r 76,000, 35,000, and 25,000 were greatest in lysates of cells treated for 24 hr with ACTH; however, the density of the band at M_r 19,000 was greatest in lysates of cells treated for 48 hr with ACTH and absent at 72 hr. The greatest density of the band at M_r 100,000 was in lysates of cells 72 hr after ACTH treatment. The scan from the lane to which the lysate of cells maintained for 24 hr in the absence of ACTH was applied is also shown for comparison. The density of the band at M_r 46,000 was greatly reduced 24 hr after ACTH treatment. ACTH also caused a number of smaller changes in the radiolabeling pattern, in particular, increases in labeling of proteins of M_r between 53,000 and

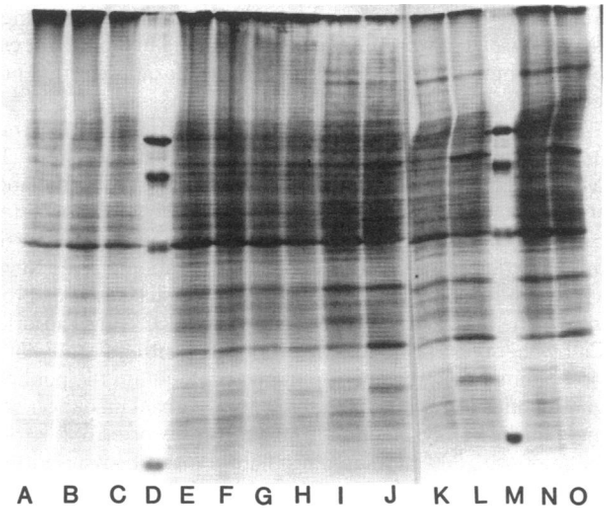

FIG. 2. Autoradiographs of [^{35}S]methionine-labeled proteins isolated from BAC cells incubated in the presence or absence of 1 μM ACTH. Lane A, 0 time, no ACTH; lane B, 2 hr, no ACTH; lane C, 2 hr, plus ACTH; lanes D and M, molecular weight standards [from top to bottom, phosphorylase b (92,500); bovine serum albumin (69,000); ovalbumin (46,000), and cytochrome *c* (12,000)]; lane E, 6 hr, no ACTH; lane F, 6 hr, plus ACTH; lane G, 10 hr, no ACTH; lane H, 10 hr, plus ACTH; lane I, 24 hr, no ACTH; lane J, 24 hr, plus ACTH; lane K, 48 hr, no ACTH; lane L, 48 hr, plus ACTH; lane N, 72 hr, no ACTH; lane O, 72 hr, plus ACTH. Molecular weight standards, radiolabeled by [^{14}C]methylation, were purchased from New England Nuclear. Samples were subjected to electrophoresis on a 12.5–7.5% gradient polyacrylamide/NaDodSO$_4$ gel.

46,000, which were incompletely resolved. This is the region in which *P*-450$_{scc}$ migrates.

Because one-dimensional NaDodSO$_4$/polyacrylamide gel electrophoresis of a total cell lysate showed too many proteins to permit resolution of *P*-450$_{scc}$, we analyzed mitochondrial protein extracts prepared from [^{35}S]methionine-labeled cells. The results of gel analysis of mitochondrial proteins from cells exposed to ACTH for 24 hr are compared with those of untreated control and those of total radiolabeled cellular protein in Fig. 4. As was the case with the whole cell radiolabeling studies, ACTH increased the radiolabeling of some mitochondrial proteins and decreased the radiolabeling of others. In particular, ACTH increased the incorporation of [^{35}S]methionine into a protein that comigrated with purified *P*-450$_{scc}$ (compare lanes C and D in Fig. 4).

The electrophoretic mobility of the *P*-450$_{scc}$ isolated from BAC mitochondria (mature form) and that of the newly synthesized *P*-450$_{scc}$ immunoisolated from a cell-free translation system by using total RNA extracted from adrenal cortical tissue are compared in Fig. 5. Clearly, the newly synthesized form is larger than the mature form. By using molecular weight standards, the molecular weights of the mature form of *P*-450$_{scc}$ and of the newly synthesized form were found to be 49,000 and 54,500, respectively. By using total RNA isolated from BAC cells maintained in monolayer culture in the presence of ACTH, a precursor of similar size was found (data not shown).

Table 1. Incorporation of [^{35}S]methionine into total adrenal cell proteins

Time, hr	ACTH	Activity, cpm/μg of protein
0	−	598
2	+	1600
6	−	2100
6	+	1400
10	−	2400
10	+	2700
14	−	2100
14	+	2600
18	+	1900
24	−	2400
24	+	2400

DISCUSSION

BAC cells in monolayer culture grow and divide in the presence of fibroblast growth factor and maintain differentiated function in the presence of ACTH and, therefore, constitute a useful model system for the study of cellular regulatory mechanisms and tropic hormone action in adult, differentiated, non-neoplastic mammalian cells. Furthermore, it is evident from the results of this investigation and of others (22–25) that the synthesis of several proteins is regulated by ACTH and that control of the synthesis of these proteins is an important component of the mechanism of action of this tropic hormone. *P*-450$_{scc}$, like

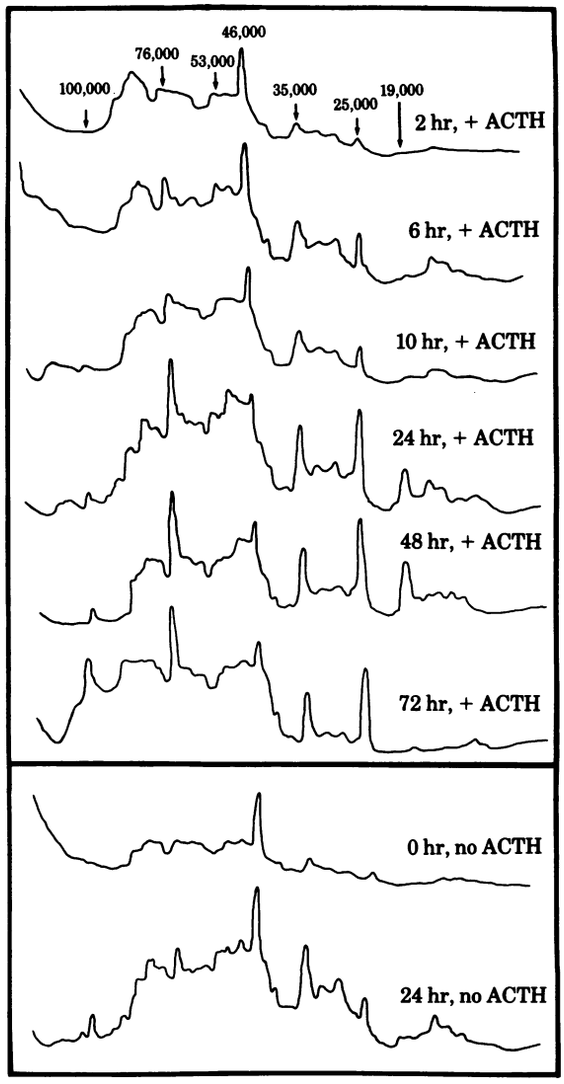

FIG. 3. Densitometric scans obtained by using a Transidyne 2955 scanning densitometer of autoradiograph lanes shown in Fig. 2. The top of the gel is at left. Molecular weights of proteins in the major peaks are marked at the top.

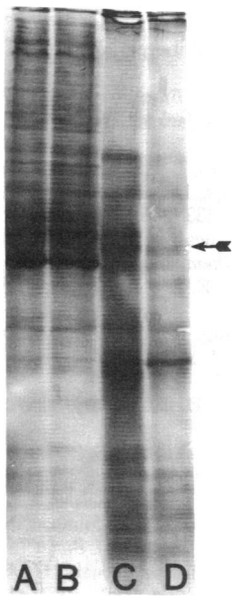

FIG. 4. Autoradiograph of [^{35}S]methionine-labeled mitochondrial proteins isolated from BAC cells maintained for 24 hr in the presence or absence of ACTH. Total radiolabeled cellular protein from these cells is shown for comparison. Lane A, total protein from cells incubated for 24 hr in the absence of ACTH; lane B, total protein from cells incubated for 24 hr in the presence of ACTH; lane C, mitochondrial protein from cells incubated for 24 hr in the presence of ACTH; lane D, mitochondrial protein from cells incubated for 24 hr in the absence of ACTH. Purified $P\text{-}450_{scc}$ migrated to the point indicated by the arrow. An increase in radiolabeling is seen at this point in lane C compared with that at the same point in lane D. Samples were subjected to electrophoresis on a 10% $NaDodSO_4$/polyacrylamide gel.

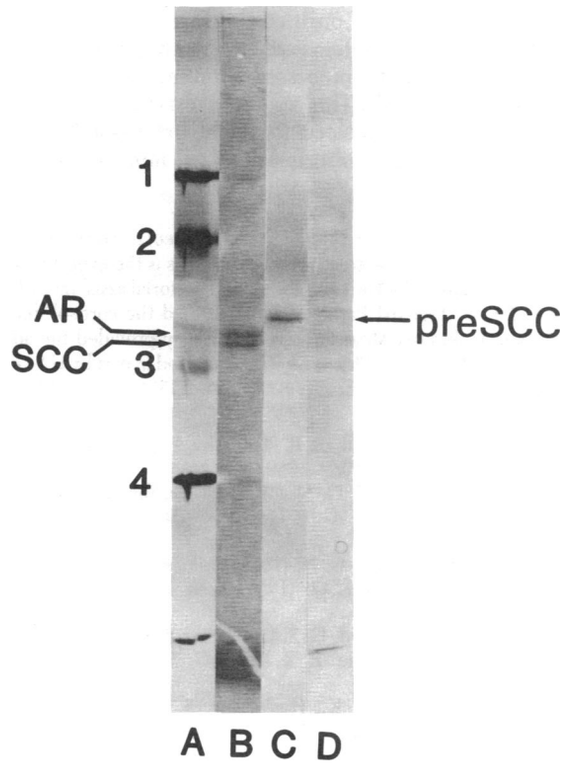

FIG. 5. Electrophoretic comparison between newly synthesized $P\text{-}450_{scc}$ immunoisolated from a cell-free translation system and the mature form isolated from BAC mitochondria. Lane A, autoradiogram of [^{14}C]methylated molecular weight standards [1, phosphorylase b (92,500); 2, bovine serum albumin (69,000); 3, ovalbumin (46,000); 4, carbonic anhydrase (30,000)]. Lane B, Coomassie blue-stained mature proteins isolated and purified from bovine adrenal mitochondria. AR, adrenodoxin reductase (M_r 51,500); SCC, $P\text{-}450_{scc}$ (M_r 49,000). The two proteins were purified independently and mixed together before electrophoresis to illustrate the M_r resolution of the gel system. Lane C, autoradiogram of newly synthesized $P\text{-}450_{scc}$ immunoisolated from the total translation products generated in the cell-free translation system. Protein synthesis was directed by total adrenal cortical RNA in the presence of [^{35}S]methionine. The immunoisolated $P\text{-}450_{scc}$ designated "preSCC" is clearly of higher M_r than the Coomassie blue-stained SCC in lane B. Lane D, immunoisolation from cell-free translation system containing no exogenous RNA. All faint bands in lane C comigrated with bands in lane D, which suggests that they are not related to the presence of adrenal cortical RNA. Samples were subjected to electrophoresis on a 12.5–7.5% gradient polyacrylamide/$NaDodSO_4$ gel.

adrenodoxin and adrenodoxin reductase, is a mitochondrial protein required for the rate-limiting step in the biosynthesis of steroid hormones from cholesterol. It is therefore likely that the synthesis of one or all of these proteins is regulated by ACTH. ACTH stimulates the cholesterol side-chain-cleavage reaction within a matter of seconds (26, 27). Such stimulation may be regarded as the acute action of this hormone and is believed to involve increased association of cholesterol and $P\text{-}450_{scc}$ within the mitochondrion (28, 29). However, ACTH also is essential for maintaining the normal function of the adrenal cortex and little is known concerning its inductive effect on the proteins required for the cholesterol side-chain-cleavage reaction. In adrenal glands of hypophysectomized rats, the concentrations of mitochondrial *P*-450 and adrenodoxin decrease with a half-life of about 4 days (30). Concomitantly, the activities of cholesterol side-chain cleavage and of steroid 11β-hydroxylation, another mitochondrial *P*-450-dependent hydroxylation reaction, decay with a similar half-life. If ACTH is administered to such animals, these activities and the content of adrenal mitochondrial *P*-450 are gradually restored to control levels over a period of days (30).

We have investigated whether ACTH treatment might induce the synthesis of $P\text{-}450_{scc}$ in BAC cells in monolayer culture, a system that is much easier to manipulate than whole animals. ACTH administration stimulated cortisol production 4-fold in these cells (see Fig. 1). In addition, the total content of spectrophotometrically detectable *P*-450 was increased about 5-fold after ACTH stimulation. More detailed experiments will be necessary to define whether ACTH preferentially affects one or more of the several different forms of *P*-450 present in these adrenal cells; however, it is reasonable to presume that $P\text{-}450_{scc}$ represents a substantial portion of the total *P*-450 (31). From these results, therefore, we conclude that $P\text{-}450_{scc}$ is likely induced by chronic ACTH stimulation.

ACTH caused a number of changes in the pattern of cellular protein radiolabeling by [^{35}S]methionine (see Figs. 2 and 3). There was increased radiolabeling in some proteins and decreased radiolabeling in others after ACTH treatment. One of the changes that occurred was an increase in a mitochondrial radiolabeled protein that comigrated with purified $P\text{-}450_{scc}$ (see Fig. 4). This result provides strong evidence that $P\text{-}450_{scc}$ is induced by ACTH. It is obvious from Fig. 4 that other mitochondrial proteins are also induced by ACTH treatment and that the inductive effect of ACTH is not specific for $P\text{-}450_{scc}$.

When total RNA isolated from either BAC cells in monolayer culture or from adrenal tissue enriched for cortical cells was used in the rabbit reticulocyte lysate system to direct synthesis of $P\text{-}450_{scc}$ and this was immunoisolated from the total translation products, the immunoprecipitated product was found to have a molecular weight of 54,500, which is 5500 greater than

the molecular weight of purified cytochrome $P\text{-}450_{scc}$. Several other mitochondrial proteins are also synthesized in precursor form and subsequently processed upon incorporation into the mitochondrion (32–37). This result suggests that $P\text{-}450_{scc}$ is synthesized as a higher molecular weight precursor that is processed before or upon insertion into the inner mitochondrial membrane.

The advice of Dr. Edward Neuwelt with respect to raising antibodies to cytochrome $P\text{-}450_{scc}$ is greatly appreciated, as is the expert technical assistance of Pamela Pickard and the skilled editorial assistance of Vicki Rankin. Dr. C. Richard Parker, Jr., performed the cortisol determinations and Columbia Meat Packers generously provided the adrenal glands used in this study. This work was supported in part by U.S. Public Health Service Grants GM27151, HD13234, HD11149, and AM27373.

1. Gospodarowicz, D., Ill, C. R., Hornsby, P. J. & Gill, G. N. (1977) *Endocrinology* **100**, 1080–1089.
2. Hornsby, P. J. & Gill, G. N. (1977) *J. Clin. Invest.* **60**, 342–352.
3. Simonian, M. H. & Gill, G. N. (1979) *Endocrinology* **104**, 588–595.
4. Hornsby, P. J. & Gill, G. N. (1978) *Endocrinology* **102**, 926–936.
5. Stone, D. & Hechter, O. (1954) *Arch. Biochem. Biophys.* **51**, 457–469.
6. Simpson, E. R. & Boyd, G. S. (1967) *Eur. J. Biochem.* **2**, 275–285.
7. Jefcoate, C. R., Hume, R. & Boyd, G. S. (1970) *FEBS Lett.* **9**, 41–44.
8. Takemori, S., Suhara, K., Hashimoto, S., Hashimoto, M., Sato, H., Gomi, T. & Katagiri, M. (1975) *Biochem. Biophys. Res. Commun.* **67**, 1151–1157.
9. Yago, N., Kobayashi, S., Sekiyama, S., Kurokawa, H., Iwai, V., Suzuki, I. & Ichii, S. (1970) *J. Biochem.* **68**, 775–783.
10. Simpson, E. R. & Boyd, G. S. (1967) *Biochem. Biophys. Res. Commun.* **28**, 945–950.
11. Bryson, M. J. & Sweat, M. L. (1968) *J. Biol. Chem.* **243**, 2799–2804.
12. Gomez-Sanchez, C., Milewich, L. & Holland, O. B. (1977) *J. Lab. Clin. Med.* **89**, 902–909.
13. Omura, T. & Sato, R. (1964) *J. Biol. Chem.* **239**, 2370–2378.
14. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
15. Kuno, H. & Kihara, H. K. (1967) *Nature (London)* **215**, 974–975.
16. Deeley, R. G., Gordon, J. I., Burns, A. T. H., Mullinix, K. P., Bina-Stein, M. & Goldberger, R. F. (1977) *J. Biol. Chem.* **252**, 8310–8319.
17. Palmiter, R. D. (1974) *Biochemistry* **13**, 3606–3615.
18. Pelham, H. R. B. & Jackson, R. J. (1976) *Eur. J. Biochem.* **67**, 247–256.
19. Ivarie, R. D. & Jones, P. P. (1979) *Anal. Biochem.* **97**, 24–35.
20. Neuwelt, E., Stumpf, D., Austin, J. & Kohler, P. (1971) *Biochim. Biophys. Acta* **236**, 333–346.
21. Seybert, D. W., Lancaster, J. R., Jr., Lambeth, J. D. & Kamin, H. (1979) *J. Biol. Chem.* **254**, 12088–12098.
22. Kowal, J., Simpson, E. R. & Estabrook, R. W. (1970) *J. Biol. Chem.* **245**, 2438–2443.
23. Asano, K. & Harding, B. W. (1976) *Endocrinology* **99**, 977–987.
24. Ray, D. B., Horst, I. A. & Kowal, J. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 4648–4652.
25. Dazord, A., Gallet, D. & Daez, J. M. (1978) *Biochem. J.* **176**, 233–239.
26. Sayers, G., Beall, R. J. & Seelig, S. (1972) *Science* **175**, 1131–1133.
27. Richardson, M. E. & Schulster, D. (1972) *J. Endocrinol.* **55**, 127–139.
28. Paul, D. P., Gallant, S., Orme-Johnson, N. R., Orme-Johnson, W. H. & Brownie, A. C. (1976) *J. Biol. Chem.* **251**, 7120–7126.
29. Brownie, A. C., Alfano, J., Jefcoate, C. R., Orme-Johnson, W. H., Beinert, H. & Simpson, E. R. (1973) *Ann. N.Y. Acad. Sci.* **212**, 344–360.
30. Purvis, J. L., Canick, J. A., Mason, J. I., Estabrook, R. W. & McCarthy, J. L. (1973) *Ann. N.Y. Acad. Sci.* **212**, 319–342.
31. Simpson, E. R. & Williams-Smith, D. L. (1976) *Biochim. Biophys. Acta* **449**, 59–71.
32. Maccecchini, M-L., Rudin, Y., Blobel, G. & Schatz, G. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 343–347.
33. Coté, C., Solioz, M. & Schatz, G. (1979) *J. Biol. Chem.* **254**, 1437–1439.
34. Shore, G. C., Carignan, P. & Raymond, Y. (1979) *J. Biol. Chem.* **254**, 3141–3144.
35. Maccecchini, M., Rudin, Y. & Schatz, G. (1979) *J. Biol. Chem.* **254**, 7468–7471.
36. Lewin, A. S., Gregor, I., Mason, T. L., Nelson, N. & Schatz, G. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 3998–4002.
37. Schmelzer, E. & Heinrich, P. C. (1980) *J. Biol. Chem.* **255**, 7503–7506.