Acute Adrenocorticotropin Hormone Stimulation of Adrenal Corticosteroidogenesis

DISCOVERY OF A RAPIDLY INDUCED PROTEIN*

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From the †Department of Biochemistry and Pharmacology, Tufts University School of Medicine, Boston, Massachusetts 02111, the §Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 and the †Mary</p><p>Ingraham Bunting Institute of Radcliffe College, Cambridge, Massachusetts 02139</p><p>Two-dimensional electrophoretic techniques were used to identify and characterize a protein that is not produced in quiescent isolated rat adrenal cells but is produced in response to acute stimulation by adrenocorticotropic hormone (ACTH) or dibutyryl cyclic AMP. The molecular weight of this protein is 28,000 (sodium dodecyl sulfate electrophoresis), and its isoelectric point is 6.5 (isoelectric focusing). Mapping of proteolytic peptides suggests that this induced protein (i) is quite similar in primary structure to another protein (p), which is produced only in nonstimulated adrenal cells. The time course of formation of protein i and its ACTH dose response closely parallel the increase in corticosteroid production in stimulated cells. The possibility that protein i is produced in response to increased levels of some steroid of the glucocorticoid pathway is precluded by the observation that inhibition of corticosteroid synthesis by aminogluthethimide does not alter the rate of production of i. Addition of cycloheximide before ACTH, which prevents stimulation of corticosteroidogenesis, also prevents formation of protein i implying that the production of protein i depends on continuing protein synthesis. [35S/32S]Methionine pulse-chase experiments, i.e., addition of excess [35S]methionine and ACTH after prelabeling with [35S]methionine, show that protein i is not produced from pre-existing protein p or other pre-existing proteins even if protein synthesis (and increased steroid production) is not inhibited. These findings exclude post-translational modification as a mechanism for the production of protein i. The fact that stimulation of corticosteroid production by aminogluthethimide does not alter the rate of production of i is consistent with the idea that protein i is not produced by the degradation of another protein. The kinetics of this stimulation have been investigated in a variety of adrenal systems including intact glands (9, 10), tissue slices (11), and dispersed cell preparations (12). It was noted here that longer term, i.e., hours or days, administration of ACTH in these systems produces additional physiological effects, which are probably of a different nature than the initial acute stereogenic response and occur with a much longer time course.

Acute ACTH stimulation (1) of adrenal corticosteroid production is caused largely by the cAMP-mediated (2-4) increase in the rate of conversion of cholesterol to pregnenolone and isopropylaldehyde (5, 6). This initial (7, 8) and rate limiting (6) step of the biosynthetic pathway is catalyzed by an adrenocortical mitochondrial enzyme system consisting of a flavoprotein (NADPH:adrenodoxin reductase); a 2Fe-2S ferredoxin (adrenodoxin), which accepts electrons from the flavoprotein; and cytochrome P-450α, which is reduced by adrenodoxin and is the site of oxygen and steroid binding and catalysis.

The kinetics of this stimulation have been investigated in a variety of adrenal systems including intact glands (9, 10), tissue slices (11), and dispersed cell preparations (12). It was found that ACTH stimulates steroid production after a lag of approximately 3 min and that subsequent removal of ACTH leads to a return to the nonstimulated rate of corticosteroidogenesis with a half-time of approximately 5–10 min. It should be noted here that longer term, i.e., hours or days, administration of ACTH in these systems produces additional physiological effects, which are probably of a different nature than the initial acute stereogenic response and occur with a much longer time course.

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† Supported by United States Public Health Service Postdoctoral Fellowship AM 06263.

‡ Supported by United States Public Health Service Research Grant AM-32455 (formerly AM-27381) and by a fellowship funded by an Office of Naval Research Grant N00014-80-C-0084 (Office of Naval Research Grant Authority Identification Number NR 390-001/2-22-80) to Radcliffe College.

1 The abbreviations used are: ACTH, adrenocorticotropic hormone; cholesterol, 5-cholesterol-3β-ol; pregnenolone, 5-pregn-3β-ol-20-one; P-450α, cytochrome P-450 from adrenal cortical mitochondria which catalyzes cholesterol side chain cleavage, i.e., the conversion of cholesterol into pregnenolone and isopropylaldehyde; P-450α, cytochrome P-450 from adrenal cortical mitochondria which catalyzes the conversion of deoxycorticosterone (4-pregn-21-ol-3,20-dione).
The sensitivity of ACTH stimulation to several different protein synthesis inhibitors has been reported. Using adrenal slices, Ferguson (13) observed that puromycin prevents not only ACTH but also cAMP stimulation of corticosteroidogenesis, which implies a site of inhibition subsequent to the formation of cAMP. Analogous results were obtained by several groups using cycloheximide both with adrenal slices (2) and in vivo (14). It was demonstrated that prior administration of cycloheximide prevented ACTH- or cAMP-mediated stimulation, that subsequent administration of cycloheximide reversed ACTH- or cAMP-mediated stimulation, and that removal of cycloheximide in both cases allowed stimulation to occur again (2, 14, 15). These findings led to the postulation of an induced stimulatory protein and its designation as the “labile protein factor”, since even after stimulation by ACTH, but without continued protein synthesis, the rate of corticosteroidogenesis rapidly returned to its unstimulated level. More recently a study by Crivello and Jefcoate (16) of five protein synthesis inhibitors of different structure demonstrated a high degree of correlation between inhibition of protein synthesis and inhibition of ACTH stimulation of steroid-hormone production.

Several attempts have been made to demonstrate synthesis of a specific protein during acute ACTH stimulation of adrenal cells, but until now no protein whose appearance is synchonized with the increase in corticosteroid production has been reported. Growdon and Brownie (17) detected a protein increase in a cytosolic protein in mouse Y-1 adrenal tumor cells as early as 15 min after addition of ACTH. Synthesis of specific proteins has also been observed for intermediate time and chronic ACTH stimulation. A low molecular weight protein was detected in Y-1 cells (18), and a $M_r = 30,000$ protein was observed in adrenal slices (19) as early as 2 h after ACTH treatment. Still longer term ACTH stimulation causes synthesis in mouse tumor cells of some components of the cytochrome P-450 electron transfer chain, i.e. adrenodoxin (20, 21) and cytochrome P-450$_{11a}$ (21). However, in the case of acute ACTH stimulation, Brownie et al. (22) have shown that the specific cytochrome P-450 content of rat adrenal mitochondria is unchanged.

We report here the discovery of a protein produced during acute ACTH or $Bt$-cAMP stimulation in isolated rat adrenal cells, whose synthetic time course and ACTH dose response are similar to these responses for corticosterone production. It is of particular interest that this protein is not produced in cells preincubated with cycloheximide or as a response to increased corticosteroid levels. This protein has a very similar primary structure to another protein synthesized only in nonstimulated cells, but it is not produced from that protein by a post-translational modification. This may have important implications for the mechanism of ACTH regulation of adrenal corticosteroid production.

**EXPERIMENTAL PROCEDURES**

**Materials**—Female Holtzman rats (150–200 g) were obtained from Charles River Breeding Laboratories, Inc., collagenase and porcine ACTH from Sigma, Inc., [35S]methionine from Amer sham Corp., Staphylococcus aureus V8 protease, from Miles Labs, and $Bt$-cAMP from P-L Biochemicals.

**Cell Preparation**—Rats were ether anesthetized and killed by decapitation. Adrenal glands were removed and stored on ice in Krebs-Ringer bicarbonate (25) containing 0.5% bovine serum albumin and 0.2% glucose until they were decapsulated and sliced for collagenase digestion. All Krebs-Ringer bicarbonate solutions were equilibrated with 5% CO$_2$, 95% air prior to use. Adrenal cells were isolated as described by Ray and Streit (24) except that KRBAG was used instead of Ham’s F-10 medium. Isolated cells were washed three times by centrifugation (200 x g for 10 min at 4°C), resuspended in KRBAG, and used in 2 h or less for the experiments described below.

A 1-h incubation at 37°C prior to use was necessary to obtain low rates of corticosterone production in nonstimulated cells. Cell viability was estimated by erythrocyte exclusion by the cells (25).

**Corticosterone Measurement**—Incubations for corticosterone production were carried out with cells in KRBAG under an atmosphere of 5% CO$_2$, 95% air in Falcon plastic tubes. Cells were incubated at 37°C for 10 min at speed 3 in a New Brunswick G-76 rotary shaking water bath (approximately 90 rpm) prior to any additions. Incubations were terminated by adding an equal volume of cold (0°C) KRBAG to the cell suspensions followed by swirling the tubes in an ice-water slurry for 15 s. The cells were collected by centrifugation as above, and the supernatant assayed for corticosterone fluorometrically (26).

**Two-dimensional Gel Electrophoresis**—When isoelectric focusing was used in the first dimension, the cell pellets were prepared essentially as described by O’Farrell (27) except that, after the addition of solid urea, 2 volumes of 9.5 m urea, 2% amphotolys (pH 3–10) pH 5–7, 1:2, 5% mercaptoethanol (v/v), 8% Nonidet P-40 (28) were added. Other solubilization methods were studied (26, 29) and we adopted the procedure which minimized precipitation of protein at the top of the first dimension gel.

First dimension isoelectric focusing was performed according to O’Farrell (27), except that the amphotolys ratio was pH 3–10/pH 5–7, 1:4. First dimension NEPHGE was performed except that the amphotolys ratio was pH 5–10/pH 5–7, 1:2. 10 µg or less of protein was applied to each gel. Application of more than 10 µg of protein resulted in considerable precipitation of protein at the top of the first dimension gel. Additionally, duplicate gels run at protein loads from 5–20 µg/gel indicated that some proteins precipitated in a variable manner. Total protein was 5 × 10$^4$ per cm. The pH range of the isoelectric focusing gels was determined as described by O’Farrell (27), equilibrating gel pieces with water.

Electrophoresis in the second dimension was also performed according to O’Farrell (27). The gels were either 7.5–18% acrylamide linear gradient gels or were 10% acrylamide uniform gels. Gels were 1 mm thick, 110 mm wide, and 50 mm high. No acrylamide stacking gel was used (29); the agarose gel used to cement the first dimension gel had the same Tris concentration and pH as the stacking gel as described by O’Farrell (27).

Two-dimensional electrophoresis using SDS-polyacrylamide gel electrophoresis in the first dimension and isoelectric focusing in the second dimension (31) was utilized for Figs. 5, 7b, 8b, and 9. The SDS gel utilized for the first dimension was 11% acrylamide. Only the $M_r = 28,000$ region of the SDS gel was used for the second dimension gel. This region was localized using dicyanlated bovine carbonic anhydrase as a marker and excised with a razor blade. The proteins in the $M_r = 28,000$ region were then separated according to charge by isoelectric focusing (ampholyte composition 1.2% pH 5–7, 0.8% pH 6–8).

Radioactivity in the gels was detected by fluorography (29), and film spot densities were converted to counts/min (27).

Protein was determined by the microbiuret method (32). Determination of [35S]methionine soluble pools and incorporation of [35S]methionine into bulk protein was according to Rodrigues and Yates (33).

Some of the proteins separated by the two-dimensional gel electrophoresis system were analyzed by partial proteolytic digestion according to the procedure of Cleveland et al. (34). Protein spots were located on the gel by salicylate fluorography (35) without prior fixation in acetic acid. The desired part of the gel was excised with a razor blade and soaked with two changes in 200 volumes each of 0.125 M Tris–Cl, pH 7.8, containing 0.1% SDS and 1.0 mM ethylenediaminetetraacetate for 20 min/change. The prepared gel pieces were fixed in acetic acid. The desired part of the gel was localized using dansylated bovine carbonic anhydrase as a marker and excised with a razor blade. The proteins in the $M_r = 28,000$ region were then separated according to charge by isoelectric focusing (ampholyte composition 1.2% pH 5–7, 0.8% pH 6–8).

**RESULTS**

**Properties of Isolated Rat Adrenal Cells**—The isolation procedure yields 1.1–2.3 × 10$^7$ viable cells/rat adrenal gland, predominantly from the zona fasciculata–reticularis (36). These cells produce corticosterone as the primary steroid in response to ACTH stimulation (37) with a maximal rate of 11 ng of corticosterone/min/10$^4$ cells (46 ng/min/adrenal equivalent). Corticosterone production is linear for at least 40 min at concentrations as high as 5.0 × 10$^6$ viable cells/ml. The primary contaminants of these adrenal cell suspensions are erythrocytes and leukocytes, which make up...
15% and less than 1%, respectively, of the total viable cells by number as determined by light microscopy.

These adrenal cells incorporate [35S]methionine into the total threochloroacetic acid-soluble pool with kinetics which approximate first order with a t1/2 of ~6 min; these pools are greater than 90% saturated 20 min after the addition of the labeled amino acid. The experiments shown in Figs. 1–4, 7a, and 8a were performed after preincubation of cells for at least 20 min with [35S]methionine.

Protein synthesis in these cells is completely inhibited within 1 min by addition of 0.2 mM cycloheximide with no decrease in cell viability up to at least 30 min. Further, cells that have been exposed to 0.2 mM cycloheximide for 20 min can reinitiate protein synthesis after removal of cycloheximide at a rate that approximates that of cells not exposed to cycloheximide.

Detection of a Protein Induced by ACTH—The following experiments were conducted to detect a protein whose formation is induced in adrenal cells by ACTH stimulation. Four aliquots of adrenal cell suspension were incubated with [35S]methionine for 23 min. Aliquot A was maintained as a nonstimulated control. Cycloheximide was added to aliquot D, and all aliquots incubated for an additional 2 min. ACTH was then added to aliquots B and D, and Bt2cAMP was added to aliquot C. The incubations were quenched 10 min later, and samples were analyzed by two-dimensional electrophoresis using isoelectric focusing in the first dimension. As shown in Fig. 1, a protein (i) with Mr ~ 28,000 is present in cells stimulated with ACTH or Bt2cAMP and not in control cells or cells treated with cycloheximide before ACTH. The letters p and n designate two proteins that are present in nonstimulated cells and have the same molecular weight (estimated from mobility on SDS gels) but different charge from protein i.

The appearance of protein i parallels the increased rate of corticosterone production, because both are induced by ACTH or by Bt2cAMP and both are inhibited by cycloheximide. This latter result suggests that the formation of this induced protein is dependent on translation (see below).

NEPHGE was used as the separation method in the first dimension to detect basic proteins that migrate out of gels run with isoelectric focusing in the first dimension (30). Fig. 2 shows results from control samples and from samples stimulated with ACTH for 30 min; no major changes are observed in the basic region of the gels. Since isoelectric focusing in the first dimension provided better resolution in the area of the induced protein, it was used in all experiments where the film was scanned to estimate the amount of radiolabel.

Having established the essential phenomenon, that this protein is produced in a cycloheximide-sensitive manner sub-

![Figure 1](http://www.jbc.org/pic/fig1.jpg)

**Fig. 1.** Two-dimensional gel electrophoresis using isoelectric focusing of rat adrenal cortical cell proteins. At t = -25 min, [35S]methionine was added (to 80 μCi/ml) to adrenal cell suspensions (4 × 10⁵ viable cells/ml) at 37 °C in a shaking water bath. The cells in A are nonstimulated controls. At t = -2 min, cycloheximide was added (to 0.2 mM) to the cells in D. At t = 0, ACTH was added (to 0.2 μM) to cells in B and D, and Bt2cAMP was added (to 2 mM) to cells in C. At t = +10 min, the incubations were quenched by addition of an equal volume of cold (4 °C) KRBAG followed by swirling the tubes in an ice-water slush for 15 s, and the samples (10 μg of protein/gel) were subjected to two-dimensional electrophoresis as described under "Experimental Procedures." Gels were prepared for fluorography and exposed for 48 h at -85 °C. Positions of molecular weight marker proteins are shown on the left side of gel A and the pH gradient of the first dimension gel, determined as described under "Experimental Procedures," is shown along the top of gel A. The induced protein is directly below the letter i (B and C), and the arrow (A and D) marks the position of molecular weight and isoelectric point of i. Protein n is above and to the right of the letter n, and protein p is directly above the letter p.
sequent to ACTH or Bt,cAMP stimulation, we next correlated aspects of its appearance with those of stimulation of corticosterone production. These aspects were the time course of formation and the ACTH dose response.

**Time Course of Induced Protein Production**—To measure the time course of formation of the induced protein, samples were incubated for different times using maximally stimulating (3, 33) concentrations of ACTH or Bt,cAMP, and electrophoresis was carried out as described in Fig. 1. The samples were also analyzed for corticosterone production. The results shown in Fig. 3 indicate that production of the induced protein i parallels corticosterone production for all samples. Bt,cAMP-stimulated corticosterone production (data not shown) is similar to that for ACTH. Production of the induced protein i is detected at the earliest time measured, 5 min, for both ACTH- and Bt,cAMP-stimulated samples. Induced protein synthesis is approximately linear between 5 and 20 min; extrapolation to zero amount of this protein indicates a lag of 3–5 min. Nonstimulated controls show essentially no production of protein i or corticosterone. This experiment has been performed on three separate cell preparations with similar results. Thus, the time course of appearance of the induced protein is quite similar to that for acute ACTH stimulation of corticosterone production.

**ACTH Dose Response of Induced Protein Formation**—For the induced protein i to be involved in increasing adrenal steroid production, the protein must be formed at ACTH concentrations less than or equal to those which result in stimulation of steroidogenesis. To test this, an ACTH dose response experiment was performed in which both corticosterone and protein i production were quantitated in cells exposed to various levels of ACTH.

Adrenal cell suspensions were incubated for 20 min with [35S]methionine, then stimulated with ACTH at the concent-

**Fig. 2. Two-dimensional gel electrophoresis (using NEPHGE) of rat adrenal cortical cell proteins.** Cells were prepared as described in the legend to Fig. 1, except that incubations were quenched at t = +30 min. Gel A is the nonstimulated control. Gel B is the ACTH-stimulated sample. 2 μg of protein were applied per NEPHGE gel. Film exposure was for 124 h at −85 °C. The basic side of the gels is to the left and positions of molecular weight marker proteins are shown on the left side of gel A. The induced protein is below and to the left of the letter i in B; the arrow in A marks the position of i. Proteins n and p are directly above the appropriate letter.

**Fig. 3. Time course of formation of the ACTH-induced protein i and corticosterone production.** The samples were prepared as described in the legend to Fig. 1. The incubations were quenched at the indicated times. Samples were subjected to electrophoresis and film spot densities converted to counts/min as described under "Experimental Procedures." Isoelectric focusing was performed using 10 μg of protein/gel. Film exposure was for 13 h at −85 °C. Data is shown for [35S]methionine incorporation into the induced protein i determined in the presence of ACTH (○), control (△), and Bt,cAMP (□). Corticosterone production (ACTH (○) and control (△)) was measured as described under "Experimental Procedures." Corticosterone production with Bt,cAMP was the same as that shown with ACTH.

As in our previous experiments, the production of induced protein closely parallels production of corticosterone.

**Lack of Corticosteroid Dependence of Induced Protein Production**—Since protein i is synthesized in adrenal cells as a result of acute ACTH (or Bt,cAMP) stimulation, we wished to investigate the possibility that it might be produced as a result of increased cellular levels of some steroid in the corticosteroid pathway. Fig. 5 shows the quantitation of protein i as a function of time in ACTH-stimulated cells and in cells which are similarly stimulated with ACTH but whose steroid production was inhibited at the initial step, i.e. cholesterol conversion to pregnenolone, by the cytochrome P-450c inhibitor aminogluthethimide. There is no difference in the amount or rate of production of protein i for these two cases. Corticosterone production in the samples to which aminogluthethimide had been added was less than that in samples without ACTH (data not shown).

Since production of protein i occurs even when pregnenolone and other corticosteroid levels are not elevated, its for-
formation is not dependent upon the reactions or products of this steroid-hormone pathway. Therefore, production of protein i occurs as a result of some events dependent on CAMP production but is not dependent on biochemical events including or after the P-450 reaction. These are properties expected for the putative protein involved in stimulation of steroidogenesis.

Kinetics of [35S]Methionine Incorporation into Protein—Fig. 6 shows the results of experiments in which the incorporation of radioactive amino acid into protein was stopped by two methods. For Fig. 6a, protein synthesis was inhibited by the addition of cycloheximide after a 9.75-min incubation with [35S]methionine. For Fig. 6b, [35S]methionine was added after an ~10-min incubation of the cells with [35S]methionine (see also Ref. 49) as for Fig. 6a. The data obtained by this procedure exactly parallel those obtained by cycloheximide inhibition, i.e. incorporation of label into protein ceases in less than 1 min. This implies that the extracellular amino acids are in rapid equilibrium with the amino acid pool used for protein synthesis. Since the t1/2 for saturation of the total soluble methionine pool was approximately 6 min, the kinetic properties of amino acid transport for the total methionine pool are different from those of the methionine pool used for protein synthesis. This rapid equilibration with extracellular amino acid allows accurate quantitation of protein synthesis with no pre-equilibration with labeled amino acid (see below).

Kinetics of Disappearance of the Induced Protein—The disappearance of protein i in ACTH-stimulated cells, measured by a change in its mobility on an O'Farrell type gel, was monitored by quantitating the amount of labeled i as a function of time after the further incorporation of label into i was inhibited by the addition of cycloheximide after a 9.75-min incubation with [35S]methionine. For Fig. 6b, [35S]methionine was added after an ~10-min incubation of the cells with [35S]methionine (see also Ref. 49) as for Fig. 6a. The data obtained by this procedure exactly parallel those obtained by cycloheximide inhibition, i.e. incorporation of label into protein ceases in less than 1 min. This implies that the extracellular amino acids are in rapid equilibrium with the amino acid pool used for protein synthesis. Since the t1/2 for saturation of the total soluble methionine pool was approximately 6 min, the kinetic properties of amino acid transport for the total methionine pool are different from those of the methionine pool used for protein synthesis. This rapid equilibration with extracellular amino acid allows accurate quantitation of protein synthesis with no pre-equilibration with labeled amino acid (see below).

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translated. For Fig. 7a, adrenal cells were incubated with \(^{[35]S}\)methionine for 20 min and subsequently stimulated with ACTH for 15 min to produce a pool of radiolabeled protein \(i\). Cycloheximide was then added to one sample while a second sample was maintained as a noninhibited control. The amount of protein \(i\) and corticosterone was monitored as a function of time. The addition of cycloheximide inhibits the synthesis of both protein \(i\) and of corticosterone; however, \(i\) is not removed from the system (degraded in such a way as to change its mobility on the gel) with the same kinetics as the return of the corticosterone production rate to basal level. For Fig. 7b, ACTH and \(^{[35]S}\)methionine were added simultaneously, and after a 15-min incubation to allow the accumulation of a pool of labeled \(i\), further incorporation of radiolabeled amino acid into \(i\) was prevented by the addition of unlabeled amino acid. Fig. 7b shows that \(i\) also disappears slowly from the system even if corticosterone production (data not shown) and protein synthesis are not inhibited.

**Translation Dependence of Induced Protein Formation**—For Fig. 8a, adrenal cells were labeled for 25 min with \(^{[35]S}\)methionine; cycloheximide was then added to one of the samples and 2 min later ACTH was added to both samples. Fig. 8a shows the kinetics of corticosterone and protein \(i\) formation as a function of the length of exposure of the cells to ACTH. Clearly, the production of both \(i\) and protein \(i\) requires continued protein synthesis. For Fig. 8b, adrenal cells were labeled for 20 min with \(^{[35]S}\)methionine; \(^{[35]S}\)methionine was then added to one of the samples and ~10 s later, ACTH was added to both. Fig. 8b shows the resulting kinetics of disappearance of protein \(i\) and formation of corticosterone and protein \(i\). The amount of protein \(i\) which disappears is insufficient to account for the amount of protein \(i\) which is formed during this period. These data again point to the dependence of the synthesis of \(i\) on translation and additionally imply that \(i\) is not produced from another protein (such as \(p\)) by either a translation-dependent or a translation-independent post-translational modification. The amount of protein \(n\) is essentially constant (values from 65–83 cpm/spot) from \(t = 0\) to \(t = 15\) min.

**Comparison of the Amounts of Proteins \(p\) and \(i\) Synthesized As a Function of the Duration of ACTH Exposure**—Because proteins \(p\) and \(i\) are essentially the same molecular weight and are similar in pl (see Fig. 1), we wish to determine if there was any relationship between them. The results shown in Fig. 6, that extracellular amino acids are in rapid equilibrium with the intracellular pools used for protein synthesis, allowed us to approach this problem by correlating the amounts of proteins \(p\) and \(i\) formed for different durations of ACTH stimulation without first labeling under nonstimulated conditions.

Fig. 9 shows the amounts of proteins \(i\) and \(p\) produced as a function of duration of ACTH stimulation. The five samples (indicated by the five time points) were all exposed to \(^{[35]S}\)methionine for 20 min. The \(t = 0\) sample had no ACTH added and therefore was under nonstimulated conditions for the entire 20-min period. The \(t = 20\) min sample had ACTH added at the same time as \(^{[35]S}\)methionine and therefore was under stimulated conditions as soon as the sample had transpired. The sample would be under nonstimulated conditions for approximately 3 min and under stimulated conditions for approximately 17 min. ACTH was added to the other samples at times to give intermediate durations of stimulated conditions between these two extremes. After the incubations were terminated, the samples were analyzed for production of proteins \(p\) and \(i\).

Samples with longer durations of ACTH stimulation have more protein \(i\) (as in Fig. 3) and less protein \(p\). The slopes of the regression lines for \(i\) and \(p\) are of opposite sign and very nearly the same magnitude. From this, we conclude that under nonstimulating conditions \(p\) is produced but not \(i\) (see also Figs. 3 and 4), and under stimulating conditions \(i\) is produced but little \(p\). A probable explanation for the presence of some \(p\) in the \(t = 20\) min sample is that it was either produced...
at 5-min intervals. At $t = -20$ min, $[^{35}S]$methionine (to 125 $\mu$Ci/ml) was added to two samples and at $t = 0$, ACTH (to 0.2 $\mu$M) was added to both. $[^{35}S]$Methionine incorporation into protein i was measured for samples stimulated with ACTH (C) and those inhibited with cycloheximide before ACTH stimulation (D). Corticosterone production (ACTH (●) and cycloheximide before ACTH (■)) was measured as for Fig. 3. b, protein i is not formed from pre-existing proteins including protein p. At $t = -20$ min, $[^{35}S]$methionine (to 125 $\mu$Ci/ml) was added to two samples of adrenal cell suspension ($6.5 \times 10^6$ viable cells/ml) at 37°C in a shaking water bath. At $t = -0.2$ min, $[^{35}S]$methionine (to 0.33 mM) was added to one sample and at $t = 0$, ACTH (to 0.2 $\mu$M) was added to both. Aliquots were removed from the sample to which $[^{35}S]$methionine had been added at $t = 0$, 5, 10, and 15 min and quenched as for Fig. 1. The sample to which no $[^{35}S]$methionine had been added was quenched at $t = 15.5$ min. Incorporation of $[^{35}S]$methionine into protein i (with (□) and without (○) $[^{35}S]$methionine) and protein p (with (△) and without (●) $[^{35}S]$methionine) was quantitated as for Fig. 1. Corticosterone production (with (■) and without (●) $[^{35}S]$methionine) was quantitated as for Fig. 3.

Fig. 8. Formation of protein i depends on protein synthesis. a, cycloheximide prevents the formation of protein i. Adrenal cell suspensions were labeled with $[^{35}S]$methionine, incubations were quenched, and electrophoretic procedures and quantitation of film spot densities were all carried out as described in the legend to Fig. 1. At $t = -20$ min, cycloheximide (to 0.2 mM) was added to one of two samples and at $t = 0$, ACTH (to 0.2 $\mu$M) was added to both. $[^{35}S]$Methionine incorporation into protein i was measured for samples stimulated with ACTH (C) and those inhibited with cycloheximide before ACTH stimulation (D). Corticosterone production (ACTH (●) and cycloheximide before ACTH (■)) was measured as for Fig. 3. b, protein i is not formed from pre-existing proteins including protein p. At $t = -20$ min, $[^{35}S]$methionine (to 125 $\mu$Ci/ml) was added to two samples and at $t = 0$, ACTH (to 0.2 $\mu$M) was added to both. Aliquots were removed from the sample to which $[^{35}S]$methionine had been added at $t = 0$, 5, 10, and 15 min and quenched as for Fig. 1. The sample to which no $[^{35}S]$methionine had been added was quenched at $t = 15.5$ min. Incorporation of $[^{35}S]$methionine into protein i (with (□) and without (○) $[^{35}S]$methionine) and protein p (with (△) and without (●) $[^{35}S]$methionine) was quantitated as for Fig. 1. Corticosterone production (with (■) and without (●) $[^{35}S]$methionine) was quantitated as for Fig. 3.

Fig. 9. Quantitation of proteins p and i as a function of length of ACTH stimulation. At $t = 0$, $[^{35}S]$methionine (to a final concentration of 200 $\mu$Ci/ml) was added to each of 5 aliquots of adrenal cell suspension ($10^6$ viable cells/ml) at 37°C in a shaking water bath. ACTH (to a final concentration of 0.1 $\mu$M) was added to the first aliquot at $t = 0$, the second at $t = 5$ min, and thus in sequence at 5-min intervals. At $t = 20$ min, all incubations were quenched as described in the legend to Fig. 1. Electrophoresis was carried out and radiolabel in proteins p (○) and i (●) was quantitated as for Fig. 5.

during the lag period and/or that very small amounts of p are produced during stimulated conditions. That production of proteins i and p is mutually exclusive suggests that they may be translated from the same mRNA.

Comparison of Proteolytic Peptides from Proteins $p$, $n$, and $i$—To test the possibility that proteins i and p have similar primary structures (as would be expected for proteins produced from the same mRNA), we examined proteolytic peptides produced from these proteins as well as from protein n, which is the same molecular weight as i and p, but intermediate in pl (6.7). Techniques for proteolytic digestion of proteins obtained from polyacrylamide gels have been described by Cleveland et al. (84).

Proteins i, p, and n were separated with the O'Farrell two-dimensional gel system and located fluorographically. The gel regions containing the desired proteins were then excised with a razor blade, loaded into separate wells of an SDS polyacrylamide gel, and subjected to proteolytic cleavage with V-8 protease from S. aureus. Because the protein samples were obtained in this manner, they do not contain equal amounts of radioactivity. Fig. 10 shows that essentially identical patterns are obtained for proteins i, p, and n at each concentration of proteolytic enzyme used. At 0.008 $\mu$g of protease/well, the initial polypeptide ($M_r = 28,000$) is present as well as a...
doublet produced by proteolytic cleavage ($M_1 = 13,000$ and 11,000). At 0.04 μg of protease/well, the ratio of proteolytic doublet to starting polypeptide has increased significantly. At 0.2 μg/well, the $M_1 = 28,000$ polypeptide is completely gone, and species of $M, < 11,000$ are produced. At 1 μg of protease/well, the $M = 13,000$ proteolytic fragment is essentially absent, and larger amounts of lower molecular weight species are present. Because the proteolytic cleavage patterns obtained from proteins i, p, and n are so similar, it is likely that their primary structures are quite similar.

**DISCUSSION**

The formation of a protein in adrenal cells in response to acute ACTH stimulation has been postulated on the basis of a variety of experiments using several inhibitors of protein synthesis (2, 13-16). We have discovered an induced protein with $M_1 = 28,000$ and isoelectric point ~6.5 in isolated adrenal cells. The protein is not formed in unstimulated cells but is produced in response to acute ACTH or Bt2cAMP stimulation with the same time course and the same ACTH dose response as the increase in amount of corticosterone produced. Further, cycloheximide added to cells before ACTH prevents stimulation of corticosteroid production and the formation of this protein; this demonstrates the dependence of these processes on protein synthesis. Addition of cycloheximide to ACTH-stimulated cells causes the rate of corticosterone production to decrease and the formation of protein i to cease; however, the amount of this protein does not decrease with a $t_{1/2}$ of ~5 min but remains essentially constant for approximately 15 min. Similar results are obtained from radiolabel pulse-chase studies, which indicate that, also under stimulated conditions (increased corticosteroid production and continuing protein synthesis) protein i is degraded slowly. Protein i is not produced as a result of the increased levels of corticosteroid subsequent to ACTH action; this is demonstrated by our studies in which ACTH-stimulated cells were treated with aminoglutethimide, which completely blocks corticosteroid production by inhibiting the initial step, i.e. pregnenolone synthesis. The time course of synthesis of protein i was the same whether or not corticosteroid production was blocked.

Nonstimulated cells produced two proteins (p and n) which have approximately the same molecular weight as protein i but differ from it in pl. The similarity of the proteolytic peptide maps of these three proteins shows their close structural relationship; their functional relationship is more difficult to evaluate. Even though the data presented in Fig. 9 are not inconsistent with protein i being produced from protein p by post-translational modification, the pulse-chase experiments detailed in Fig. 8b show that this is not the case. Further, from the data shown in Fig. 9, we may conclude that if protein p is made, protein i is not and vice versa.

At present, we do not know the functions of proteins i, n, and p, however, there are certain possibilities which may be excluded. Since shunting of cholesterol to, or pregnenolone from, cytochrome P-450<sub>10</sub> might increase the rate of the enzyme reaction and since cytochrome P-450<sub>10</sub> is inhibited (41, 42) by and activated (11, 43, 44) by specific phospholipids, the stimulatory protein factor might be a sterol or phospholipid carrier protein. There are numerous reports of sterol carrier proteins (45-48); further, one study (49) showed stimulation of pregnenolone synthesis in rat adrenal mitochondria incubated with lipid droplets by addition of sterol carrier protein 2. However, none of the proteins reported thus far has a molecular weight and isoelectric point matching those of protein i. Specifically of interest are cholesterol binding protein and sterol carrier protein 2, which have $M_1 = 26,000$ and 13,500, respectively, and isoelectric points 5.8 and 8.6, respectively (46, 49), compared with 28,000 and 6.5 for protein i. Cholesterol esterase (50) and calmodulin (51), which have each been shown to stimulate adrenal corticosteroid production under certain conditions, have $M_1 = 41,000$ (50) and 16,500 (52), respectively; additionally, calmodulin has an isoelectric point of approximately 4.0 (52). The three protein components of the cholesterol side chain cleavage enzyme system also differ in monomer molecular weight, which is 51,000, 12,000, and 46,000 for the flavoprotein reductase (53), adrenodoxin (54), and cytochrome P-450<sub>10</sub> monomer, respectively. Thus, all these proteins differ significantly from i in at least one of these physical parameters and may be excluded as candidates for this rapidly induced protein. One further point, which should be mentioned here in the discussion of proteins whose function makes them potential stimulators of steroid synthesis, is the prediction made by Kido and Kimura (56) on the basis of studies using poly-L-lysine to stimulate pregnenolone synthesis in vesicles containing the proteins of the cholesterol side chain cleavage enzyme system. They suggest that the labile protein must be a basic polypeptide of $M_1 < 10,000$, both of these properties are significantly different from those of protein i.

The kinetics of disappearance of i do not correlate with those of decreased corticosteroid output after inhibition of protein synthesis, since the former process is much slower than the latter. This problem may be addressed from a slightly different point of view by asking why the postulated stimulatory effect of i is so short lived. One explanation is that i is inactivated, for example by loss of noncovalently bound cofactor or a change in configuration, by a process which does not change its mobility on an O’Farrell type gel. A second possibility is that i is used only once or a limited number of times, for example, as a sterol carrier protein which remains in the mitochondrial membrane.

At this point, the function of protein i and the nature of the modification(s) that causes it to differ in pl from n and p are both open to speculation. However, it is of particular interest that a co-translational modification mechanism accounts for several of the unusual aspects of stimulation of corticosterone production. This is the first direct evidence of the cycloheximide-sensitive production of a protein in response to acute ACTH (or Bt2cAMP) action with the same dose response and time course as stimulation of corticosterone synthesis.

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