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Biochemistry

Characterization of a common precursor to corticotropin and β-lipotropin: Cell-free synthesis of the precursor and identification of corticotropin peptides in the molecule*

(peptide analysis/messenger RNA/immunoprecipitation)

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ABSTRACT **mRNA was isolated from cultures of AtT-20/D-16v tumor cells and translated in a mRNA-dependent reticulocyte cell-free system. The corticotropin (ACTH) product was purified by a double-antibody immunoprecipitation procedure using antisera specific for the α(1–24) sequence of ACTH. The product is shown by sodium dodecyl sulfate/gel electrophoresis and gel filtration on guanidine·HCl columns to be homogeneous with an apparent molecular weight (M_r) of 28,500. A product with the same molecular weight is synthesized when membrane-bound polysomes from D-16v cells are allowed to complete their nascent chains in a reticulocyte cell-free system. M_r 31,000 ACTH isolated from tumor cells has been separated into three proteins of different apparent M_r: 29,000, 32,000, and 34,000. The cell-free product contains the same lysine-, methionine-, and phenylalanine-labeled tryptic peptides as the M_r 29,000 ACTH synthesized in the tumor cells. Tryptic peptide analysis also reveals the presence of the α(1–39) sequence in the M_r 28,500 cell-free product and suggests that there is only one copy of this sequence in the M_r 28,500 molecule.**

High molecular weight forms of corticotropin (ACTH) have been observed in extracts of pituitary glands and pituitary tumor cells (1–4). Studies of the biosynthesis of ACTH in a mouse pituitary tumor cell line (AtT-20/D-16v line) that contains four molecular weight (M_r) classes of ACTH suggest that the M_r 31,000 ACTH in these cells is a precursor of M_r 23,000, 13,000, and 4500 classes of ACTH (5). The M_r 31,000, 23,000, and 13,000 molecules are all glycosylated (6).

To investigate the initial steps in ACTH biosynthesis, mRNA was isolated from AtT-20/D-16v cells and translated in a reticulocyte cell-free system. The purified product is shown to be a homogeneous polypeptide of apparent M_r 28,500. Cell-free synthesis of ACTH has also been accomplished in two other laboratories. Translation of mRNA from bovine pituitary (7) and from a mouse pituitary tumor (8) has yielded ACTH products with M_r of 35,000 and 31,000, respectively. No attempt was made in those studies to relate the structure of the major ACTH product made to that of high molecular weight glycosylated forms of ACTH present in pituitary cells. Several approaches were used in the present study to investigate this relationship. One of these approaches, tryptic peptide analysis, also yielded information about the number of copies of the α(1–39) sequence in the cell-free product.

Interest in the synthesis and post-translational processing of high molecular weight forms of ACTH has intensified recently with the discovery that these molecules contain a β-lipotropin-like sequence in addition to α(1–39) ACTH; β-lipotropin is known to contain the opiate peptide, β-endorphin (β-lipotropin 61-91) (9). The occurrence of the tryptic peptides of β-lipotropin in the cell-free product described here and the location of these peptides relative to that of α(1–39) peptides is the subject of the second paper in this series.

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MATERIALS AND METHODS

Cell Fractionation and Preparation of Polysomes. Cells were grown to confluency in plastic roller bottles (Corning) in Dulbecco–Vogt minimal essential medium with 5% horse serum (10), washed twice with serum-free medium, scraped from the bottles, and centrifuged. The pellet was resuspended in 10 volumes of buffer A [25 mM sucrose/1.5 mM $MgCl_2$/50mM NaCl/25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.2] and homogenized with a glass–Teflon homogenizer (five strokes by hand). (Microscopic examination of the homogenate indicated that >95% of the cells had been broken by this method.) Nuclei were sedimented at 800 × *g* for 10 min. The post-nuclear supernatant was then centrifuged at 20,000 × *g* for 30 min. The 20,000 × *g* supernatant contained the free polysomes.† The pellet (particulate fraction) was resuspended in 10 volumes of buffer B (25 mM NaCl/5 mM $MgCl_2$/25 mM Hepes, pH 7.2/0.5% Nonidet P40), homogenized, and centrifuged at 20,000 × *g* for 30 min. This supernatant contained the released membrane-bound polysomes.

Free and membrane-bound polysomes were isolated by layering the supernatant fractions over a discontinuous gradient (1 ml of 60% sucrose and 2 ml of 30% sucrose, both in buffer B without detergent) and centrifuging in an SW 41 rotor at 40,000 rpm for 2 hr. The sedimented polysomes were resuspended in 20 mM Hepes, pH 7.2/5 mM $MgCl_2$ and stored at −20°. One gram of cells yielded approximately 4 mg of polysomes. Radioactive amino acid-labeled cell extracts were prepared as described (5) except that cells were grown in Dulbecco–Vogt medium with 10% horse serum.

Immunoprecipitations. Three antisera to ACTH were purified by affinity chromatography with either α(1–24) or α_p(1–39) ACTH-Sepharose (5). Antiserum Bertha, which was used for the bulk of the experiments, is specific to the α(11–24) region of ACTH (10). The other antisera are specific for

Abbreviations: ACTH, corticotropin; M_r, molecular weight; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; TPCK, L-1-tosylamido-2-phenylethyl chloromethylketone.

* This is the first of a series of two papers on the common precursor. The second paper is on the lipotropin portion of the molecule.

† Free ribosomes in the 20,000 × *g* supernatant give a typical polysome profile in a sucrose density gradient without prior treatment with detergent, whereas ribosomes in the particulate fraction (20,000 × *g* pellet) give a typical polysome profile only after treatment with detergent. Ribosomes in the particulate fraction sediment to the bottom of the gradient if not treated with detergent.

α_p(25–39) ACTH (which crossreacts with mouse ACTH) and α(7–18) ACTH. A double-antibody immunoprecipitation procedure was used with both cell extracts and reticulocyte lysates. The lysates were diluted with 2 volumes of immunoprecipitation buffer (buffer E, ref. 5) made 1% (vol/vol) in Triton X-100 (Amersham/Searle) before addition of the antiserum.

RNA Preparation. RNA was extracted from the post-nuclear supernatant of cell homogenates by phenol/$CHCl_3$ extraction (11). The RNA was treated with autodigested Pronase (Sigma) and precipitated with LiCl (12). The precipitate was dissolved in H_2O and precipitated with 2 volumes of ethanol and 0.1 volume of 4 M NaCl. The RNA precipitate was stored at −70° in 20 mM Hepes, pH 7.2. One gram of cells gave approximately 2 mg of cytoplasmic RNA. Poly(AMP)RNA was purified from the cytoplasmic RNA by poly(UMP)-Sepharose (Pharmacia) affinity chromatography (13).

Protein Synthesizing Systems. Rabbit reticulocyte lysates were prepared and stored under liquid N_2 as described (14). After inactivation of endogenous mRNA by nuclease treatment (15), incorporation of labeled amino acids into trichloroacetic acid-precipitable material was completely dependent upon added mRNA. The components of the lysate mixture are those described by Woodward *et al.* (14). Cytoplasmic RNA was added to a final concentration of 100 μg/ml, and polysomes were added to a final concentration of 200 μg/ml. Both concentrations were in the linear portion of the dose-response curve for added RNA concentration versus trichloroacetic acid-precipitable radioactivity. Aurin tricarboxylic acid, an inhibitor of protein chain initiation (16) at 0.1 mM, was included in the lysate incubations with polysomes to allow only the completion of nascent chains. The lysate mixtures (230 μl) were incubated at 30° for 60 min, and an aliquot was removed for determination of total incorporation (14). The remainder of the lysate was immunoprecipitated with ACTH-specific antisera.

Analysis of Labeled Immunoprecipitates. A sodium dodecyl sulfate ($NaDodSO_4$)/10% acrylamide gel (borate/acetate, pH 8.5; ref 5) and a $NaDodSO_4$/12% acrylamide gel (Biophore gel system, Bio-Rad; Tris/acetate, pH 6.3; ref. 17) were used to analyze immunoprecipitates. Immunoprecipitates were solubilized by boiling in a gel buffer (diluted 1:5 with H_2O, made 8 M in urea, 1% in $NaDodSO_4$, and 5% in 2-mercaptoethanol). Electrophoresis was at 8 mA per gel. Gels were cut into 1-mm slices (Bio-Rad gel slicer), incubated in 300 μl of 0.1% $NaDodSO_4$/0.1% Triton X-100/50 mM Tris, pH 7.6 at 37° for 16 hr. An aliquot was assayed for radioactivity to locate the labeled proteins. Appropriate fractions were pooled, 100 μg of bovine serum albumin was added, and the protein was precipitated by addition of trichloroacetic acid to 10% (wt/vol). The precipitate was centrifuged and the pellet was washed twice with acetone/ether, 1:1 (vol/vol). This material was used for analyses on $NaDodSO_4$ gels, by tryptic digestion, or by gel filtration on Bio-Gel A-1.5m in 6 M guanidinium chloride/bovine serum albumin, 0.2 mg/ml. Residual $NaDodSO_4$ in the protein pellets did not affect either the gel filtration or the tryptic digest analyses.

Analysis of Tryptic Digests of Labeled ACTH Proteins. Radioactive ACTH proteins from either cell extracts or reticulocyte lysates containing 100 μg of bovine serum albumin were dissolved in 200 μl of 0.1 M NH_4HCO_3, pH 8.5; 5 μg of L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Worthington) was added and the mixture was incubated at 37° with shaking. Another 5 μg of the trypsin was added at 4–6 hr. After 18–20 hr, the reaction was stopped by freezing the mixture. After lyophilization, peptides were dissolved in 1% acetic acid and analyzed by paper electrophoresis at pH 6.5 (pyridine/H_2O/acetic acid, 10:90:0.35, vol/vol), and descending paper chromatography (*n*-butanol/acetic acid/H_2O, 4:1:5, vol/vol, upper phase) (18).

RESULTS

Intracellular Location and Synthesis of ACTH on Membrane-Bound Polysomes. If a cell fractionation is performed as described above, 96% of the radioimmunoassayable ACTH present in the homogenate is found in the particulate fraction composed mainly of mitochondria, secretory granules, and reticulum.

To determine which class of ribosomes synthesizes ACTH, free and membrane-bound polysomes were isolated by using a differential centrifugation procedure.† The absorbance ratio (A_{260}/A_{280}) was 1.8 for both classes of polysomes. Membrane-bound polysomes comprised ~80% of the total polysomal material present in the cells. Free and membrane-bound ribosomes showed similar A_{260} profiles after sedimentation in a 15–30% (wt/vol) sucrose gradient with most of the ribosomes present as large polysomes (more than five ribosomes) and less than 5% present as monoribosomes.†

Polysomes were allowed to complete their nascent chains in a reticulocyte or a wheat germ cell-free protein-synthesizing system in the presence of radioactive amino acids. The amount of radioactivity incorporated into trichloroacetic acid-insoluble material (per A_{260} unit of polysomes) was the same for both classes of polysomes. The ACTH-synthesizing capability of the polysomes was measured by immunoprecipitation of the labeled lysate mixtures. The immunoprecipitates were solubilized and assayed for radioactivity. Only the membrane-bound polysomes showed immunoprecipitable radioactivity, accounting for 2–5% of the total trichloroacetic acid-precipitable radioactivity, depending upon the radioactive amino acid used.

Analysis of the Size of ACTH Made under the Direction of Tumor Cell RNA and by Allowing Polysomes to Complete Nascent Chains. RNA was isolated by phenol/$CHCl_3$ extraction of the post-nuclear fraction of tumor cells. In some cases, the RNA was further fractionated by poly(UMP)-Sepharose chromatography, yielding a poly(AMP)-enriched RNA fraction that accounted for 1.8% of the total cytoplasmic RNA. Cytoplasmic RNA and the poly(AMP)-enriched RNA were translated in a modified reticulocyte lysate, a wheat germ lysate (19), or the *Xenopus* oocyte (20). The reticulocyte system translated either form of RNA efficiently, but the other systems required poly(AMP)-enriched RNA for efficient translation. The proteins synthesized in these systems were labeled with various radioactive amino acids and isolated by immunoprecipitation with three purified ACTH antisera. The immunoprecipitable radioactivity was 4–8% of the trichloroacetic acid-precipitable radioactivity in the reticulocyte lysate. The M_r distribution of labeled proteins in the immunoprecipitate was analyzed by two $NaDodSO_4$/polyacrylamide gel systems and by gel filtration in 6 M guanidinium chloride (Fig. 1). With the reticulocyte system, only one radioactive peak was seen on $NaDodSO_4$ gels with an apparent molecular weight of 28,500 ± 600 (mean ± SD, n = 26). Specificity of antiserum Bertha is shown by the absence of labeled protein in the immunoprecipitate formed in the presence of excess α(1–24) (Fig. 1*A*). Similar results were obtained with the other two antisera. When the M_r 28,500 material from the $NaDodSO_4$ gels was analyzed on a guanidinium chloride column (Fig. 1*B*), it had an apparent M_r of 28,000 ± 2,000 (n = 4). The major ACTH product made in the wheat germ or oocyte system was also a M_r 28,500 protein.

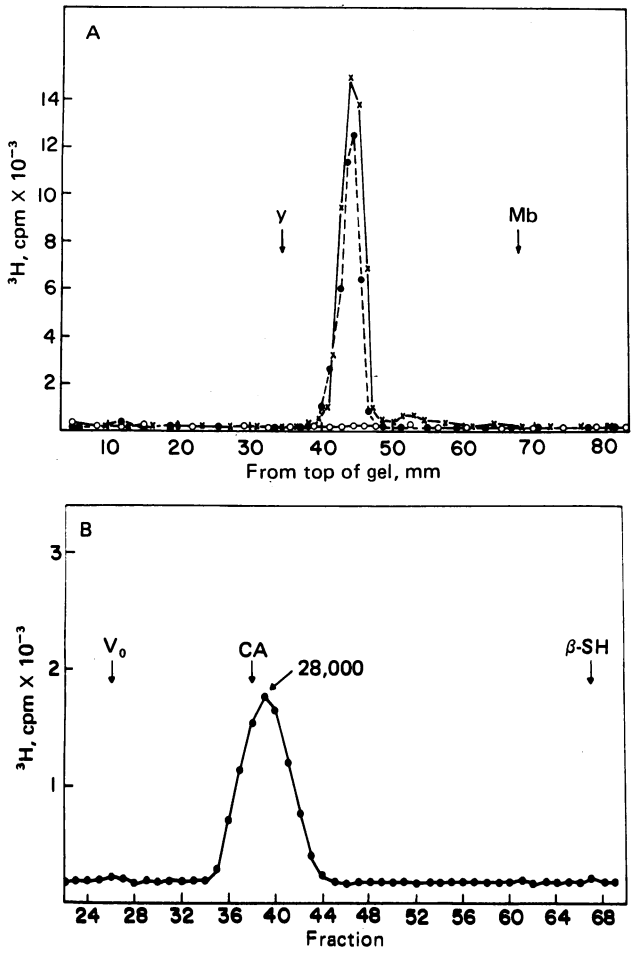

FIG. 1. Size analysis of the ACTH synthesized in the reticulocyte system. (*A*) AtT-20 RNA or polysomes were incubated in the reticulocyte lysate with [^{3}H]lysine (20 μM, 60 Ci/mmol, Amersham/-Searle). ACTH (~10 ng) was purified by immunoprecipitation and subjected to electrophoresis on a 12% Biophore gel. One-millimeter slices were cut, eluted, and analyzed for ^{3}H radioactivity in a Triton/xylene fluor by liquid scintillation counting (^{3}H counting efficiency, ~40%). Dansylated yeast alcohol dehydrogenase (y) and dansylated myoglobin (Mb) were included as internal markers. M_r was determined by running parallel gels containing ovalbumin, yeast alcohol dehydrogenase, carbonic anhydrase, chymotrypsinogen A, RNAse, and cytochrome *c* as standards and by defining the mobility of yeast alcohol dehydrogenase as 1.0. ●, ACTH immunoprecipitate of AtT-20 RNA-directed proteins; ×, ACTH immunoprecipitate from AtT-20 polysome chain completion experiment; ○, ACTH immunoprecipitate of AtT-20 RNA-directed proteins formed in the presence of excess competing antigen [10 μg of α(1–24) ACTH].

(*B*) The M_r 28,500 [^{3}H]ACTH material from a NaDodSO$_4$ gel was isolated by trichloroacetic acid precipitation and dissolved in 100 μl of 6 M guanidinium chloride/bovine serum albumin, 0.2 mg/ml. Blue dextran (Vo), carbonic anhydrase (CA), and 2-mercaptoethanol (β-SH) were added as internal markers. This solution was chromatographed on a Bio-Gel A-1.5m column (0.8 × 50 cm), and 600-μl fractions were collected. A_{280} was measured and then the entire fraction was solubilized in 6 ml of fluor and counted. M_r was assigned from a standard curve with ovalbumin, carbonic anhydrase, cytochrome *c*, and chymotrypsinogen A as markers.

However, smaller proteins were also made in the wheat germ system and a slightly larger protein M_r (~31,000) was made in the oocyte (data not shown). The reticulocyte and wheat germ systems were unable to incorporate either [^{3}H]glucosamine or [^{3}H]mannose into a trichloroacetic acid-precipitable protein. Results of similar studies with the oocyte systems were less clear.

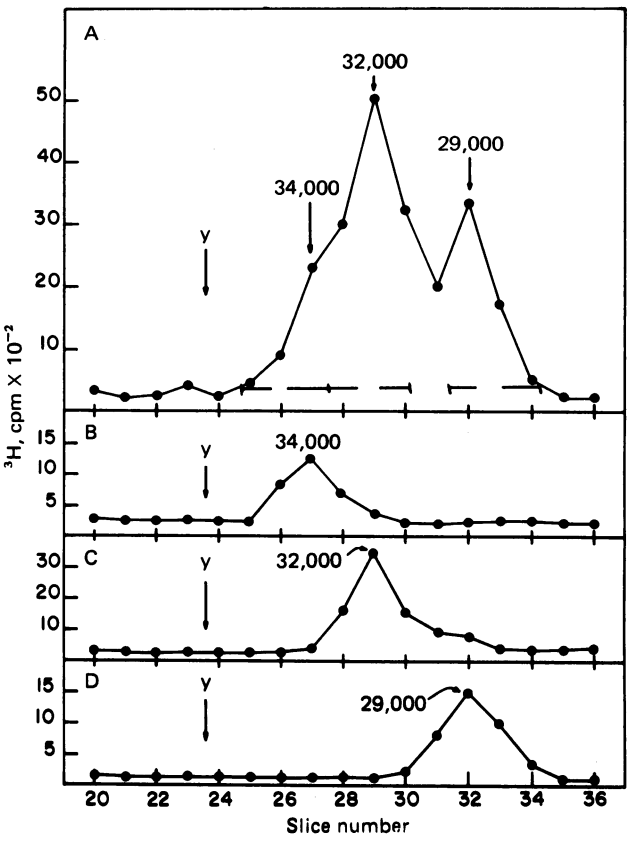

FIG. 2. Fractionation of the intact cell M_r 31,000 ACTH proteins. (*A*) Confluent cultures in Falcon Microtest wells were incubated with [4,5^{3}H]lysine (Amersham/Searle, 60 Ci/mmol, 50 μCi in 50 λ of medium) for 30 min. The cells were rinsed twice with unlabeled medium and extracted with 5 M acetic acid/bovine serum albumin, 5 mg/ml. The lyophilized extracts (35 ng of radioimmunoassayable ACTH) were subjected to electrophoresis on a 12% Biophore gel. The gels were sliced and eluted, and an aliquot of the eluate was assayed for radioactivity. The remainder of the eluate was pooled as shown (I, II, III) and protein was isolated by trichloroacetic acid precipitation. Dansylated yeast alcohol dehydrogenase (y) was an internal marker. (*B*, *C*, and *D*) Fractions I, II, and III, respectively, were subjected to electrophoresis again on 12% Biophore gels and the gels were assayed for radioactivity. M_r was determined as in Fig. 1.

AtT-20 polysomes were allowed to complete their nascent chains in the reticulocyte cell-free system. Only one size class of ACTH was synthesized with an apparent M_r of 28,500 (Fig. 1*A*).

Relationship of Cell-Free ACTH to High Molecular Weight ACTH Synthesized in Tumor Cells. It has been shown by pulse-chase experiments with tumor cells that ACTH is synthesized as a M_r 31,000 glycoprotein (6). Using the 12% Biophore NaDodSO$_4$/gel electrophoresis system, we have been able to resolve the M_r 31,000 ACTH into three proteins (M_r 34,000, 32,000, and 29,000) (Fig. 2*A*). M_r was determined by reanalyzing pooled fractions of these proteins in the same NaDodSO$_4$/gel system (Fig. 2 *B*, *C*, and *D*).

Peptide mapping of the three components (labeled with amino acids and sugars) has indicated that the M_r differences are due mainly to differences in carbohydrate content and not to differences in peptides (unpublished data). Thus, the component with the least carbohydrate (M_r 29,000 ACTH) has been used to show the relationship between peptides in intact cell ACTH and cell-free ACTH (which has no carbohydrate). Gel fractions containing the cell-free product (Fig. 1*A*, fractions 42–47) and M_r 29,000 ACTH (Fig. 2*D*) were pooled, eluted,

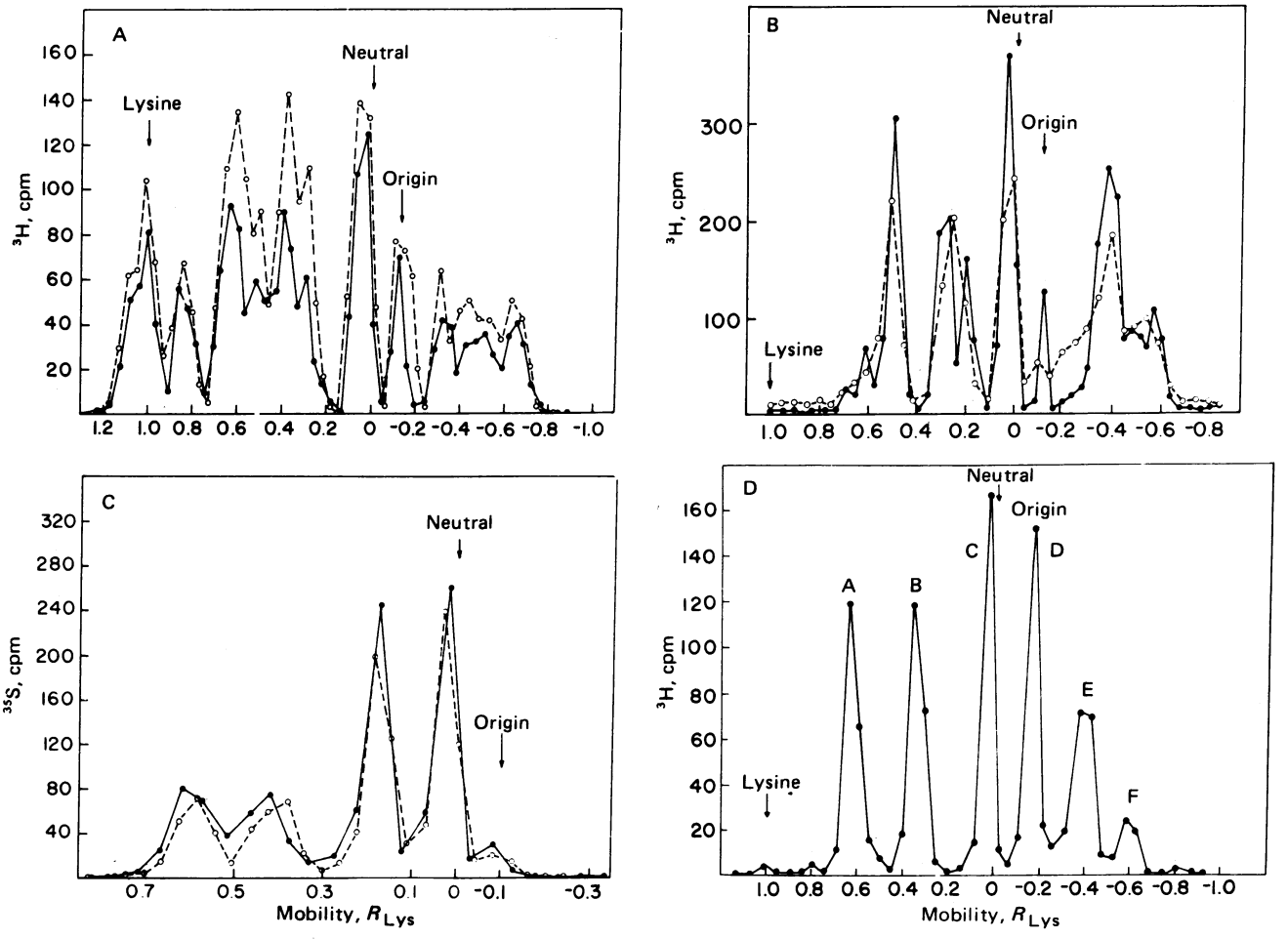

FIG. 3. Paper electrophoresis of tryptic peptides at pH 6.5. After radioactive cell-free M_r 28,500 ACTH was isolated and subjected to electrophoresis as in Fig. 1, it was eluted from the gel and precipitated with trichloroacetic acid. The washed precipitate was dissolved in 100 μl of 0.1 M NH_4HCO_3, pH 8.5, treated with TPCK-trypsin, and lyophilized. The lyophilized peptides were dissolved in 1% acetic acid, spotted on strips of Whatman no. 1 paper (ε-DNP-lysine, lysine, and picric acid were included as internal markers), and subjected to electrophoresis at 2000 V/50 cm for ~1.5 hr. The strips were sprayed with ninhydrin to locate lysine and ε-DNP-lysine, cut into 8-mm slices, and soaked in scintillation vials with 500 μl of 0.1% $NaDodSO_4$/0.5 M urea for 2 hr. Six milliliters of fluor was added and the vials were assayed for radioactivity. A background of 12 cpm has been subtracted from all data points. Mobility was defined relative to lysine, with ε-DNP-lysine taken as $R_{Lys} = 0$ and lysine as $R_{Lys} = 1$. An identical procedure was used to analyze the tryptic peptides of cell extract M_r 28,000–29,000 ACTH (fraction III, Fig. 2). The radioactive labels used were: [^{3}H]lysine (60 Ci/mmol) (*A*), [^{3}H]phenylalanine (27 Ci/mmol) (*B*), [^{35}S]methionine (510 Ci/mmol) (*C*), and [^{3}H]tryptophan (23 Ci/mmol) (*D*); all were from Amersham/Searle. (*A*), [^{3}H]lysine; (*B*), [^{3}H]phenylalanine; and (*C*), [^{35}S]methionine. In *A*, *B*, and *C*: ●, cell-free M_r 28,500 ACTH; ○, cell-extract M_r 28,000–29,000 ACTH. In *D*: ●, cell-free M_r 28,500 ACTH. The percentage of total radioactivity recovered under each peak in *D* was as follows: A, 18.5%; B, 19.9%; C, 21.2%; D, 19.6%; E, 16.4%; and F, 4.3%.

digested with trypsin, and subjected to paper electrophoresis at pH 6.5. Comparison of [^{3}H]lysine-, [^{3}H]methionine-, and [^{3}H]phenylalanine-labeled peptides showed that the cell-free product contained the same tryptic peptides as the AtT-20 M_r 29,000 ACTH (Fig. 3 *A*, *B*, and *C*).

Identification of the α(1–21) ACTH Peptides in Cell-Free ACTH. To substantiate that the cell-free product contained the α(1–39) sequence, a search was made for α(1–39) peptides in tryptic digests of the cell-free product. Tryptic peptides from porcine ACTH were used as markers, because the α(1–24) sequence is known to be conserved in mammals (21).

The α(1–39) tryptic peptides were separated by paper electrophoresis at pH 6.5, located with ninhydrin, and identified by amino acid analysis except for α(22–39) (Table 1).

To identify ACTH tryptic peptides in the cell-free product, the molecule was labeled with each of 16 amino acids, and peptide profiles were generated for each amino acid. By analyzing the quantity of radioactivity present in a peak with a specific mobility, a partial amino acid composition of the tryptic fragments was determined. An example of this analysis with [^{3}H]tryptophan is shown in Fig. 3*D*. There are six radioactive peaks, four of equal size and the sum of the remaining two equals each of the other four. Because chromatography in a second dimension indicated that the tryptophan-labeled peptides were homogeneous, the simplest interpretation is that there are five tryptophan-containing peptides (one of which exhibits variable tryptic cleavage), each having only one tryptophan residue. One of the labeled tryptophan peptides ($R_{Lys} = 0.61$, peak A in Fig. 3*D*) comigrated with the α(9–15) ACTH peptide known to have only one tryptophan residue (Table 1). The profile in Fig. 3*C* suggests that there are only three methionine-containing peptides present, one of which is the α(1–8) peptide ($R_{Lys} = 0.18$) and one of which exhibits variable tryptic cleavage (chromatography of the material in the peak containing the α(1–8) peptide revealed only one methionine peptide). Similar analysis with 13 other amino acids provided the basis for the identification of the amino acids in Table 1. Two-dimensional analyses of methionine- and phenylalanine-labeled peptides will be presented in the second paper in this series.

Table 1. Partial amino acid composition and electrophoretic mobilities of α(1–21) tryptic peptides from the cell-free product

ACTH peptides		R_{Lys}*	
		α_p(1–39) marker peptides	Labeled peak in cell-free ACTH tryptic profile
1–8	Ser-Tyr-Ser-Met-Glu-His-Phe-Arg†	0.15 – 0.18	0.15 – 0.21
9–15	Trp-Gly-Lys-Pro-Val-Gly-Lys	0.59 – 0.63	0.58 – 0.67
16	Lys	0.98 – 1.02	0.97 – 1.02
17	Arg	0.91 – 0.94	0.90 – 0.97
16–17	Lys-Arg	1.07 – 1.10	1.05 – 1.12
18–21	Arg-Pro-Val-Lys	0.82 – 0.84	0.79 – 0.88

* Mobilities relative to lysine were determined from the center of the radioactive or ninhydrin spot. The range of values is for mobilities obtained in at least three different electrophoresis runs.

† A two-dimensional separation of this peptide will be shown in the second paper in this series.

DISCUSSION

The results show that ACTH is like other secretory proteins (22); it is made on membrane-bound ribosomes and immediately sequestered in the endoplasmic reticulum (particulate material).

RNA and polyribosomes both directed the synthesis of only one ACTH protein in the reticulocyte system with an apparent M_r of 28,500. Studies with the tumor cells have shown that M_r 29,000 ACTH is the first of the three components of M_r 31,000 ACTH to be synthesized (unpublished data); therefore, we used this protein to determine the relationship of the cell-free synthesized ACTH to the intact cell ACTH. Fig. 3 *A–C* shows that M_r 29,000 ACTH has the same peptide content as the cell-free product.

Several laboratories have recently reported the cell-free synthesis of secretory proteins in the wheat germ cell-free protein-synthesizing system (23–27). The synthesized proteins differed from the corresponding protein isolated from intact cells in that they contained an additional 15 to 30 amino acids at their amino terminus (pre-sequence). Sequencing work is needed to determine if a pre-sequence is present in the primary transcript of ACTH.

The results in Fig. 3*D* show that there are five tryptophan peptides present in about equal amounts in the RNA-directed cell-free product. One of the peptides [α(9–15)] is derived from the α(1–39) region of the M_r 28,500 molecule. The same analysis can be done for the three methionine-containing peptides in this product. [There is only one tryptophan and one methionine residue in mouse α(1–39) (unpublished data).] The simplest interpretation of these results is that there is only one copy of each of these α(1–39) peptides in the M_r 28,500 molecule and, therefore, one copy of the α(1–39) sequence.

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