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Research Article

This study was designed to establish definitively the nature of immunoreactive lipotropin (IR-LPH) in human plasma and tissue extracts. Using gel filtration, gel filtration under denaturing conditions, cationic exchange chromatography, immunoprecipitation, and radioimmunoassay, we have studied normal and tumorous human pituitaries, ectopic ACTH- and LPH-secreting tumors, plasma from normal subjects before and after dexamethasone administration, and plasma from patients with primary adrenal insufficiency and pituitary and nonpituitary ACTH- and LPH-secreting tumors. Except in the plasma and tumors of occasional patients with ectopic ACTH syndrome, the smallest IR-LPH appears to be λ-lipotropin (λLPH), which is often the predominant and occasionally the only IR-LPH present. The other major peptide appears to be βLPH, a 91-amino acid molecule that contains λLPH as its 1-58 sequence. Larger immunoreactive materials were observed in some specimens, but the "big" LPH in one plasma was shown to be λLPH bound to IgG.

The weak melanocyte-stimulating activity of LPH suggests that ACTH may be the principal pigmentary hormone in man. The fact that λLPH, rather than βLPH, is the predominant form in plasma suggests that the enkephalin-endorphin opiate peptides, which are contained in the "missing" 59-91 sequence from the βLPH precursor of λLPH, may be secreted in parallel with ACTH under both physiological and pathological conditions in man.

The Nature of the Immunoreactive Lipotropins in Human Plasma and Tissue Extracts

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ABSTRACT This study was designed to establish definitively the nature of immunoreactive lipotropin (IR-LPH) in human plasma and tissue extracts. Using gel filtration, gel filtration under denaturing conditions, cationic exchange chromatography, immunoprecipitation, and radioimmunoassay, we have studied normal and tumorous human pituitaries, ectopic ACTH- and LPH-secreting tumors, plasma from normal subjects before and after dexamethasone administration, and plasma from patients with primary adrenal insufficiency and pituitary and nonpituitary ACTH- and LPH-secreting tumors. Except in the plasma and tumors of occasional patients with ectopic ACTH syndrome, the smallest IR-LPH appears to be γ-lipotropin (γLPH), which is often the predominant and occasionally the only IR-LPH present. The other major peptide appears to be βLPH, a 91-amino acid molecule that contains γLPH as its 1–58 sequence. Larger immunoreactive materials were observed in some specimens, but the "big" LPH in one plasma was shown to be γLPH bound to IgG.

The weak melanocyte-stimulating activity of LPH suggests that ACTH may be the principal pigmentary hormone in man. The fact that γLPH, rather than βLPH, is the predominant form in plasma suggests that the enkephalin-endorphin opiate peptides, which are contained in the "missing" 59–91 sequence from the βLPH precursor of γLPH, may be secreted in parallel with ACTH under both physiological and pathological conditions in man.

INTRODUCTION

The radioimmunoassay (RIA)[1] studies of Abe et al. (1–3) appeared to establish that the 22 amino acid polypeptide, human β-melanocyte-stimulating hormone (hβMSH) (4) was the principal pigmentary hormone in man. However, Scott and Lowry (5) and Bloomfield et al. (6) reinvestigated the nature of immunoreactive (IR) hβMSH and were unable to demonstrate a molecule of this size in extracts of normal human pituitary or in unextracted plasma from two patients with pituitary tumors and progressive hyperpigmentation after total bilateral adrenalectomy for Cushing's disease (Nelson's syndrome). Instead, they found that most IR-βMSH corresponded in size to β-lipotropin (βLPH), a 91 amino acid molecule whose 37–58 sequence is identical with that of hβMSH (7) with lesser amounts corresponding in size to γLPH ([1–58]βLPH) in pituitary extracts (5) but not in plasma (6). Therefore, they questioned the existence of hβMSH and presented evidence that it was an artifact produced by enzymic degradation of βLPH during the weak acid extraction originally employed for isolating hβMSH from human pituitaries (8). These observations have been confirmed by ourselves and others (9–12). However, although our preliminary studies (9–11) and the published work of others (12) suggested that βLPH was the smallest IR-LPH in human plasma and tissue, we more recently found evidence that γLPH concentrations might equal or exceed those of βLPH in many human plasma and tissue specimens. The potential importance of this distinction is that the 61–91 sequence of βLPH contains the endogenous opiate-like peptides, methionine-enkephalin (13) and the endorphins (14, 15). Thus, if a major circulating LPH were γLPH, the opiate peptides contained in the remaining 59–91 sequence of the βLPH precursor molecule might also circulate in plasma and possibly exert their effects on a variety of target tissues.

In this study, the apparent molecular sizes of IR-LPH in unextracted human plasma and extracts of plasma and tissues were characterized by gel filtration and compared with those of highly purified reference hβLPH and hγLPH.

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[1] *Abbreviations used in this paper*: Gdn, guanidine; IR, immunoreactive; K_d, fractional elution volume; LPH, lipotropin; MSH, melanocyte-stimulating hormone.

METHODS

Preparation of samples

Pituitary tissue. A single normal whole human pituitary gland obtained at autopsy and fresh-frozen whole human pituitary glands provided by the National Pituitary Agency were stored at −70°C until they were extracted. The frozen glands were homogenized in ice-cold glacial acetic acid (19 ml/g tissue) by three 15-s bursts in a Waring blender (Waring Products Div., Dynamics Corp. of America, New Hartford, Conn.), cooling the contents of the blender cup in an ice bath between bursts. The homogenate was centrifuged at 6,000 *g* for 20 min at 4°C, and the supernate was stored at −70°C.

Pituitary tumor culture medium. A pituitary tumor causing Nelson's syndrome (progressive hyperpigmentation after bilateral total adrenalectomy as treatment for Cushing's disease) was obtained at surgery. The tumor cells were dispersed and cultured at 37°C in Dulbecco's modified Eagle's minimal essential medium (North American Biologicals, Inc., Miami, Fla.) containing 15% horse serum and 2.5% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.) under a 12% CO_2 atmosphere. After 2 wk of culture, during which the medium was replaced every 2–4 days, the medium was aspirated, the adherent cells were washed several times with 10–20 ml of fresh medium, and 25 ml of fresh medium was added. After an additional 3 days of incubation, the medium was aspirated, centrifuged at 6,000 *g* for 15 min at 4°C, and the supernate was carefully decanted and stored at −70°C.

Nonpituitary tumor tissue. Tumor tissues (a medullary carcinoma of the thyroid, a pancreatic islet cell carcinoma, and an epithelial thymoma) causing the ectopic ACTH syndrome were obtained from three patients at surgery and homogenized immediately or after storage at −70°C in ice-cold deionized, glass-distilled water (9.0 ml/g tissue); the supernate was prepared and stored as described above.

Plasma. Lightly heparinized blood was obtained from normal subjects, one patient with untreated primary adrenal insufficiency (Addison's disease), four patients with Nelson's syndrome, and three patients with ectopic ACTH syndrome. Endogenous IR-LPH was suppressed in normal subjects by oral administration of dexamethasone, 2 mg at bedtime and 2 mg the next morning; blood was obtained 2 h later. All blood was chilled immediately and centrifuged at 2,000 *g* for 15 min at 4°C. Plasma was aspirated, transferred to another polypropylene tube, and centrifuged at 6,000 *g* for 10 min at 4°C (16). The plasma was carefully decanted and stored at −70°C.

Gel exclusion chromatography

All chromatography was performed at 4°C. Five different columns were prepared for the purposes of this study.

Large G-50 column. This column was used for initial analyses of plasma and tissue extracts. A 5 × 90-cm column was packed with Sephadex G-50 fine resin (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.), equilibrated, and developed with 0.1 M ammonium acetate that was adjusted to pH 7.4 with 14.8 M ammonium hydroxide. Samples and calibration standards were eluted by descending flow (50 cm hydrostatic pressure, 3.8 ml/cm² per h) and 16.8-ml fractions were collected. Calibration standards were dissolved in 10 ml of column buffer, and the sample volume was 10 ml or was diluted 10 ml with the column buffer, except for 34.6 and 36.2 ml of two tumor extracts that were applied.

Small G-50 column. This column was used for studies of extraction efficiency. A 1.6 × 88-cm column was prepared in the manner described above. A 2-ml sample or calibration standard was eluted by descending flow (23 cm hydrostatic pressure, 4.5 ml/cm² per h), and 1.6-ml fractions were collected.

G-75 Column. This column was used to characterize IR-LPH in an extract of pooled human pituitaries. A 5 × 90-cm column of Sephadex G-75 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc.) was prepared as described above. 20-ml samples or standards were eluted by descending flow (50 cm hydrostatic pressure, 3.8 ml/cm² per h), and 17.0-ml fractions were collected.

G-100 Column. This column was used to characterize IR-LPH in plasma from a patient with Nelson's syndrome. A 5 × 90-cm column of Sephadex G-100 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc.) was prepared as described above. 10 ml of samples or standards was eluted by gravity flow (35 cm hydrostatic pressure, 2.7 ml/cm² per h), and 17.0-ml fractions were collected.

CL-6B/guanidine-HCl column. This column was used to characterize IR-LPH under denaturing conditions. A 1.6 × 90-cm column was packed with cross-linked agarose resin (Sepharose CL-6B, Pharmacia Fine Chemicals, Div. of Pharmacia Inc.), equilibrated, and developed with a 0.1-M phosphate buffer, pH 7.0, made 6 M with guanidine-HCl (Gdn-HCl) (Ultra-pure, Schwarz/Mann Div., Bectin, Dickinson, and Co., Orangeburg, N. Y.) (17). A 1-ml sample or standard was eluted by ascending flow (15 cm hydrostatic pressure, 1.33 ml/cm² per h) and 19-drop fractions were collected in individually preweighed polypropylene tubes containing 0.5-ml RIA standard diluent with twice the usual protein concentration to prevent the adsorption of peptides to the plastic; the actual fraction size was determined by weight (17). Each sample or standard was preincubated in a 6-M Gdn-HCl phosphate buffer for 18 h at 4°C, 3 mg of Blue Dextran 2000 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc.) and 0.1 mg of 2,4-dinitrophenyl-L-alanine (Sigma Chemical Co., St. Louis, Mo.) were added immediately before application to the CL-6B/Gdn-HCl column.

Calibration standards. The following is a list of standards with their source, molecular weight, and method of detection, in that order: synthetic human (37–58)LPH (hβMSH) (Ciba-Geigy, Ltd., Basel, Switzerland), 2,661, radioimmunoassay; purified hγLPH (Dr. P. J. Lowry), 6,300, radioimmunoassay; purified hβLPH (Dr. C. H. Li and Dr. Lowry), 10,033, radioimmunoassay; the preceding four peptides labeled with ^{125}I by modifications (16, 18) of the method of Hunter and Greenwood (19), gamma scintillation spectrometry; synthetic hACTH (Ciba-Geigy, Ltd.) labeled with ^{3}H (20) 4,541, beta scintillation spectrometry; bovine gamma globulin (Cohn fraction II, Sigma Chemical Co.) 150,000, OD_{280}; bovine serum albumin (Sigma Chemical Co.) repurified on Sephadex G-100, 67,000, OD_{280}; and NaCl (Fisher Scientific Co., Pittsburgh, Pa.), 58, flame photometry. For the CL-6B/Gdn-HCl column, the following were also used: cytochrome *c* (type VI, horse heart, Sigma Chemical Co.), 12,400, OD_{405}; myoglobin (type II, whale skeletal muscle, Sigma Chemical Co.) reduced and carboxymethylated (17), 16,950, OD_{280}; ovalbumin (Sigma Chemical Co.) reduced and carboxymethylated, 43,000, OD_{280}; Blue Dextran 2000, 2,000,000, OD_{630}; and 2,4-dinitrophenyl-L-alanine, 255, OD_{360}.

Cation-exchange chromatography

A 1.5-liter glacial acetic acid extract of fresh-frozen human pituitary glands (75 g, ≅150 glands) was diluted to 7.5 liters

with distilled water, the pH was adjusted to 3.0 with 17.5 M NaOH, and it was chromatographed on a 2.6 × 90-cm column of Amberlite CG-50 (Rohm and Haas Co., Philadelphia, Pa.) at 64 ml/cm² per h (21). The fraction eluted with 1.15 liters of 60% acetic acid, which contained the IR-LPH, was then diluted with water, lyophilized, reconstituted in a 0.1-M ammonium acetate buffer, pH 7.4, and subjected to G-75 gel-exclusion chromatography.

Immunoprecipitation

The nature of the high molecular weight IR-LPH in the plasma of a patient with Nelson's syndrome was investigated using plasma from a patient with Addison's disease as a control. Approximately 2.5 ng of freshly repurified ^{125}I-hγLPH (Dr. Lowry) was incubated for 48 h at 4°C with 0.3 ml of each plasma diluted to 2.5 ml with the RIA standard diluent. Total protein-bound ^{125}I counts in 0.1 ml of the incubation mixtures were precipitated with 1.5 ml of 20% polyethlene glycol (Carbowax 6000, Union Carbide Corp., Chemical and plastics, N. Y.) (wt/vol) in the RIA buffer (18, 22). The ^{125}I counts bound to specific plasma immunoglobulins were precipitated by adding graded volumes of polyvalent anti-human immunoglobulin serum and monospecific anti-human IgG, anti-human IgM, and anti-human IgA (Meloy Laboratories Inc., Springfield, Va.) to equal 0.1-ml aliquots of the incubation mixtures, incubating them an additional 24 h at 4°C, adding 2 ml of RIA standard diluent, and centrifuging the tubes at 5,000 *g* for 20 min at 4°C; the supernates were carefully decanted and the pellets were counted in a gamma scintillation spectrometer.

LPH radioimmunoassay

Procedure. The RIA for LPH was performed (16, 23) using an antiserum, G-106, whose characteristics have been described (23). This antiserum was raised[2] in a guinea pig injected subcutaneously with commercial porcine ACTH (Corticotrophin-Zinc, 50 U/ml, Organon Inc., West Orange, N. J.) which is heavily contaminated with porcine MSH, a molecule which shares the 47–58 amino acid sequence of human βLPH and γLPH (16, 23). Purified hγLPH (Dr. Lowry) and hβLPH (Dr. Li and Dr. Lowry) generated displacement curves parallel to synthetic (37–58)hLPH standard (Ciba-Geigy Ltd.) and, on a molar basis, were 82 and 58% as effective, respectively, as the synthetic LPH fragment in competing with ^{125}I-(37–58)hLPH for antibody-binding sites (23); all results are expressed as weight equivalents of synthetic (37–58)hLPH standard. The antibody demonstrated <0.1% cross-reactivity with either synthetic α-MSH or hACTH (both from Ciba-Geigy Ltd.). Thus, this is a (47–58)hLPH RIA. Two studies were performed with an antiserum, R-3, raised in a rabbit injected intradermally with synthetic (37–58)hLPH covalently bound to bovine serum albumin, which demonstrated similar specificity (23). The results of this LPH RIA are also expressed as weight equivalents of synthetic (37–58)hLPH standard.

Preparation of samples. Some samples with very high IR-LPH concentrations were diluted in RIA standard diluent and assayed directly. Fractions from the G-50, G-75, and G-100 columns were lyophilized, stored at −70°C, and reconstituted in the RIA standard diluent immediately before assay. The CL-6B/Gdn-HCl column fractions were desalted on 1.1 × 26-cm Sephadex G-10 columns. 1 mg of Blue Dextran 2000 in 0.1 ml of 5% acetic acid was added to each fraction, which was then applied and eluted with 5% acetic acid by descending flow (82 cm hydrostatic pressure, 32 ml/cm² per h); 1.0-ml fractions were collected. The fractions containing Blue Dextran 2000 were pooled, lyophilized, stored at −70°C, and reconstituted in the RIA standard diluent just before assay.

[2] Abe, K., and D. N. Orth. Unpublished data.

RESULTS

IR-LPH in normal pituitary gland extracts. The relative efficiency of three methods of extracting IR-LPH from pooled fresh-frozen human pituitary glands was evaluated. Extraction into 2.5% HCl-97.5% acetone (vol/vol) (24) yielded 81.0±1.5 (SE) μg/g frozen tissue, water extraction (25) yielded 143.0±1.8 μg/g, and glacial acetic acid (26) extracted 210.0±6.1 μg/g. The glacial acetic acid procedure was chosen. Fig. 1A shows the elution profile of a single human pituitary gland

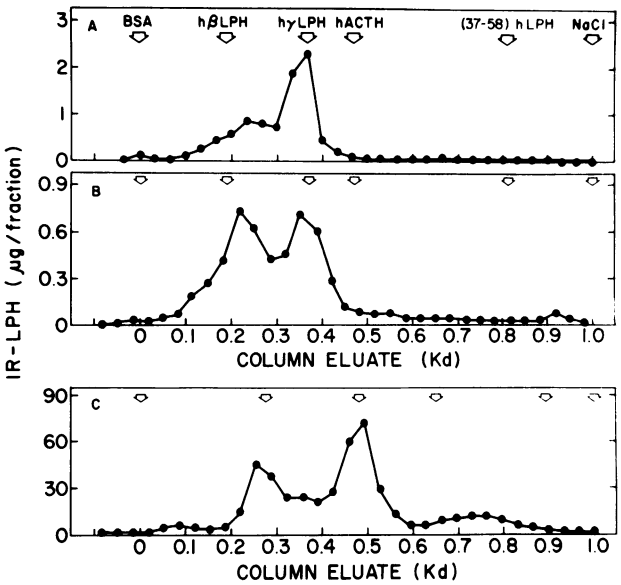

FIGURE 1 Elution profiles of human pituitary extracts from Sephadex columns. (A) Glacial acetic acid extract of a single pituitary gland on the large Sephadex G-50 column (performed with the R-3 antiserum). The IR-LPH concentration in alternate 17.0-ml fractions is plotted. The elution positions of bovine serum albumin (BSA, void volume, 646 ml), NaCl (total volume, 1,666 ml), purified hβLPH (Dr. Li), hγLPH (Dr. Lowry), synthetic (1–39)hACTH, and (37–58)hLPH (hβMSH) are indicated. Elution volume is plotted as a fraction (K_d) of column volume, and IR-LPH as micrograms of (37–58) hLPH/fraction. Recovery of total IR-LPH from the large G-50 column in all experiments was 89.5 ±6.8% (SE). (B) Glacial acetic acid extract of ≈150 fresh-frozen human pituitary glands (National Pituitary Agency) under identical conditions. (C) Sephadex G-75 gel filtration of a glacial acetic acid pituitary extract after Amberlite CG-50 cationic exchange chromatography. Column dimensions and buffer were the same as for the large G-50 column, and the same calibration standards are shown in the same order. Sample volume, was 20-ml; fraction volume, 17.0 ml; void volume, 663 ml and total volume, 2,363 ml.

extract on a Sephadex G-50 column. IR-LPH eluted in two peaks (the fractional elution volume [K_d] = 0.23 and 0.37) corresponding closely with those of purified hβLPH and hγLPH (K_d = 0.19 and 0.37, respectively). Fig. 1B depicts the elution pattern of the extract of pooled human pituitary glands. The pattern was similar, except that the size of the two peaks was nearly identical. Fig. 1C shows the elution profile of the Amberlite CG-50 extract (21) applied to Sephadex G-75. The pattern was again similar, with two distinct peaks in positions corresponding to those of β- and γLPH. No IR-LPH the size of hβMSH was detected.

Thus, two components, β- and γLPH, accounted for essentially all of the IR-LPH in these extracts, and concentrations of γLPH appeared to be equal to or greater than those of βLPH.

Plasma IR-LPH in patients with pituitary LPH hypersecretion. Unextracted plasma from four patients with Nelson's syndrome was subjected to Sephadex G-50 gel filtration (Fig. 2A–D). In each, the smallest IR-LPH peak had the apparent molecular size (K_d = 0.34 – 0.40) of γLPH. In each, the concentration of γLPH exceeded that of βLPH, which was virtually absent in two of them (Fig. 2C, D). In three of the plasmas, IR-LPH was observed in the column void volume, representing a major component of one of them (Fig. 2B).

The IR-LPH secreted by pituitary tumors causing Nelson's syndrome were also characterized by culturing tumor cells and examining the medium in which they were cultured. Three major IR-LPH peaks were observed (Fig. 2E), one in the void volume, one (K_d = 0.20) similar in molecular size to βLPH, and one (K_d = 0.40) that appeared to be γLPH.

Because these data were all concerned with IR-LPH secreted by pituitary tumors, unextracted plasma from a patient with a normal pituitary gland and LPH hypersecretion due to Addison's disease was examined. The major peak of IR-LPH (K_d = 0.38) appeared to be γLPH; only small amounts eluted before this single major peak (Fig. 2F).

No IR-LPH the size of hβMSH was observed in any of these specimens. Thus, β- and γLPH accounted for most of IR-LPH in most plasma specimens, and γLPH concentrations equaled or exceeded those of βLPH in each; variable amounts of still larger IR-LPH were also observed.

IR-LPH in nonpituitary tumor extracts. Because IR-LPH secretion is almost invariably associated (2) with that of ACTH by tumors causing the ectopic ACTH syndrome, we examined the IR-LPH in extracts of tumors from such patients. In each, the major IR-LPH peak (K_d = 0.36 – 0.39) coeluted with purified hγLPH (Fig. 3A–C) and, in one of the tumor extracts (Fig. 3C), it was the only IR-LPH detected. Smaller amounts of IR-LPH coeluted (K_d = 0.16 and 0.23) with hβLPH

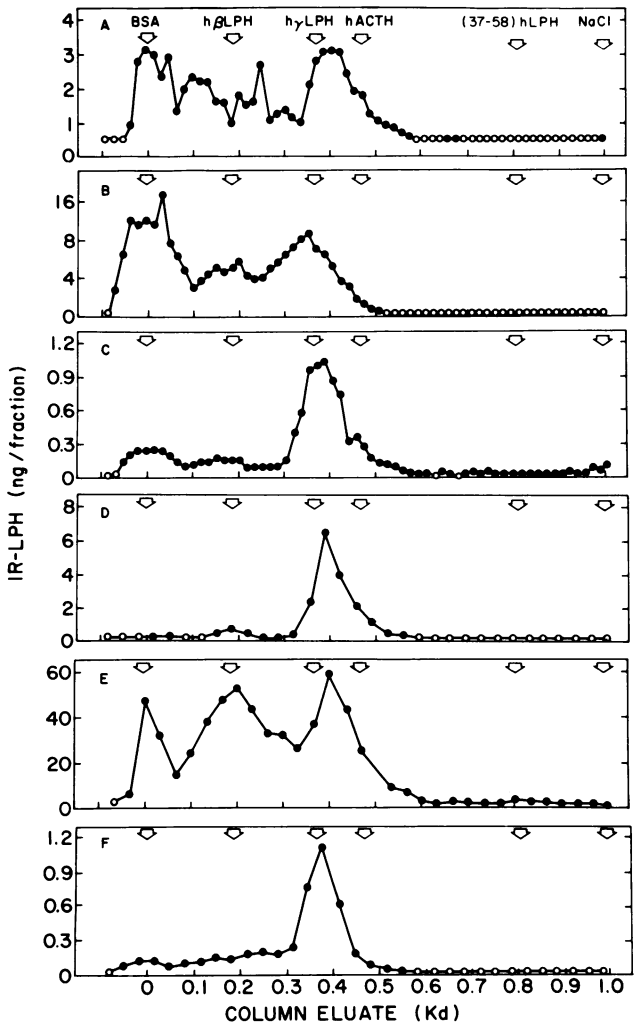

FIGURE 2 Elution profiles of plasma from patients with Nelson's syndrome and Addison's disease, and tissue culture medium from a tumor causing Nelson's syndrome from the large Sephadex G-50 column. (A–D) Unextracted plasma from four patients with Nelson's syndrome; 2.0, 2.0, 10.0, and 5.3 ml, respectively, of plasma were diluted to 10.0 ml with column buffer before application. (E) 10 ml of tissue culture medium in which a human pituitary adenoma causing Nelson's syndrome had been incubated for 48 h, 2 wk after initial explant. (F) 8.5 ml of unextracted plasma from a patient with Addison's disease, diluted to 10.0 ml with column buffer, and applied. Conditions were the same as in Fig. 1, except consecutive fractions were assayed for IR-LPH and plotted for A–C. The open circles in this and all following figures represent nondetectable IR-LPH at the concentration plotted.

in two tumors, and significant amounts of IR-LPH eluted just after the void volume (K_d = 0.01) and in a broad peak (K_d = 0.81) coeluting with hβMSH (Fig. 3A).

Thus, in nonpituitary tumors as in the normal pituitary gland, the major IR-LPH appeared to be γLPH. In contrast, however, both very large and small,

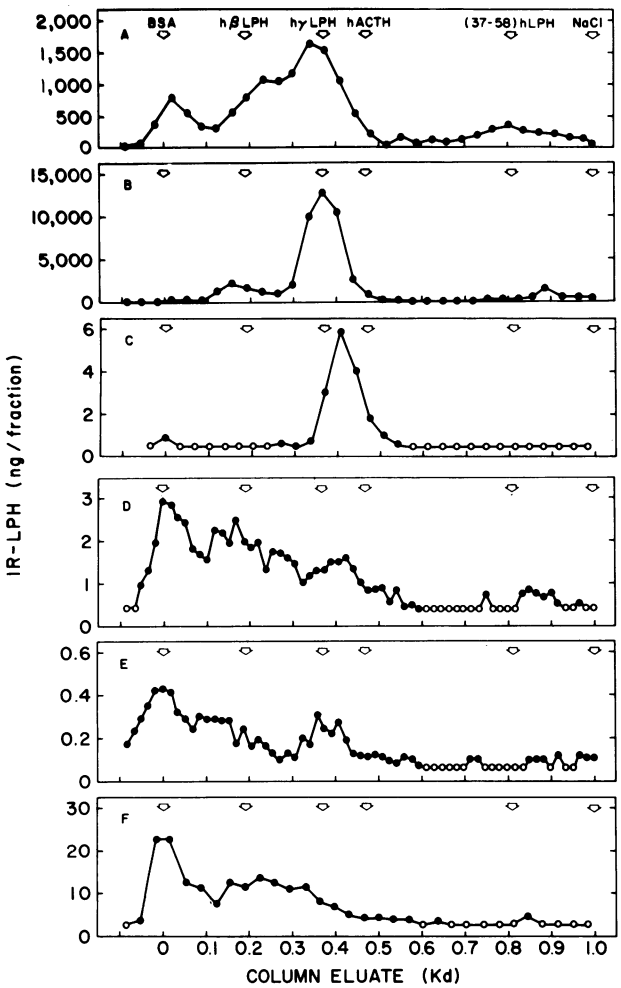

FIGURE 3 Elution profiles of tumor extracts and plasma from patients with ectopic ACTH syndrome from the large Sephadex G-50 column. (A) Epithelial thymoma; 36.2 ml of extract equivalent to 3.62 g of wet tissue applied. (B) Pancreatic islet cell carcinoma; 34.6 ml of extract equivalent to 3.46 mg of wet tissue applied. (C) Medullary carcinoma of the thyroid; 1.0 ml of extract equivalent to 35 mg of tissue was diluted to 10.0 ml with column buffer and applied. 10 ml of unextracted plasma from patients with (D) oat cell carcinoma of the lung, (E) pancreatic islet cell carcinoma (not the same patient as in Fig. 4B), and (F) oat cell carcinoma of the lung. Conditions were the same as in Fig. 1; two extracts (A,B) were assayed with R-3 antiserum; consecutive (D,E) or alternate (A–C,F) fractions were assayed and plotted.

hβMSH-sized components were sometimes present, albeit in relatively small amounts.

Plasma IR-LPH in patients with nonpituitary tumors. In unextracted plasma from each of three patients with ectopic ACTH syndrome, IR-LPH was found in fractions from the void volume through the elution volume of γLPH (Fig. 3D–F). Although high molecular weight LPH predominated, significant peaks of γLPH-sized IR-LPH were also observed in two plasmas (Fig. 3D, E), and in one, a small peak eluted just after (K_d = 0.85) synthetic hβMSH.

Thus, in contrast to patients with pituitary hypersecretion, patients with ectopic ACTH syndrome had increased amounts of heterogeneous, high molecular weight plasma IR-LPH, relatively less γLPH, and, like the tumors themselves, occasionally contained hβMSH-sized IR-LPH.

Pituitary IR-LPH under denaturing conditions. To exclude the possibility that either the β- or γLPH-sized peptide in pituitary extracts represented a smaller IR peptide aggregated or bound to a larger protein, pooled peak fractions from Sephadex G-75 gel filtration (Fig. 1C, K_d = 0.21 – 0.33 and 0.42 – 0.53, respectively) were analyzed by gel filtration in 6 M Gdn-HCl. The larger IR-LPH coeluted with hβLPH (Fig. 4A) and the smaller with hγLPH (Fig. 4B), with no conversion of either to smaller IR peptides.

Thus, the IR-LPH in human pituitary extracts appeared to represent β and γLPH, and no hβMSH was detected, as previously reported (5, 6).

Plasma IR-LPH under denaturing conditions. The three major IR-LPH peaks from Sephadex G-50 filtration of plasma from a patient with Nelson's syndrome (Fig. 2B, K_d = 0.08–0.05, 0.13–0.21, and 0.30–0.42, respectively) were analyzed by gel filtration in 6 M Gdn-HCl. Of the two smaller peaks, the larger peak (Fig. 4D) appeared to be βLPH and the smaller peak, γLPH (Fig. 4E). In contrast, the IR-LPH that had eluted in the G-50 column void volume now appeared to consist mostly of γLPH (Fig. 4C).

Thus, plasma β- and γLPH-sized IR-LPH were not smaller IR peptides aggregated or bound to other plasma proteins; it appeared that the largest IR-LPH in one plasma was γLPH bound to plasma proteins.

Plasma IR-LPH after dexamethasone administration. To ascertain if the plasma IR-LPH in the void volume was an artifact caused by high protein concentrations, three normal subjects were given dexamethasone to suppress endogenous ACTH and LPH secretion. There were no detectable IR peptides except in the void volume fractions. As in the other plasmas, void volume displacement curves were slightly less steep than that of standard (concentrations were estimated from the sample volume required to cause 50% fall in trace binding). Furthermore, the amount of void volume IR-LPH was <5% that observed in the plasma of patients with Nelson's syndrome. Because the void volume protein concentrations were presumably similar, and because some patients with Nelson's syndrome did not have appreciable void volume IR-LPH (Fig. 2D), RIA interference by plasma proteins seemed an unlikely explanation for void volume IR-LPH.

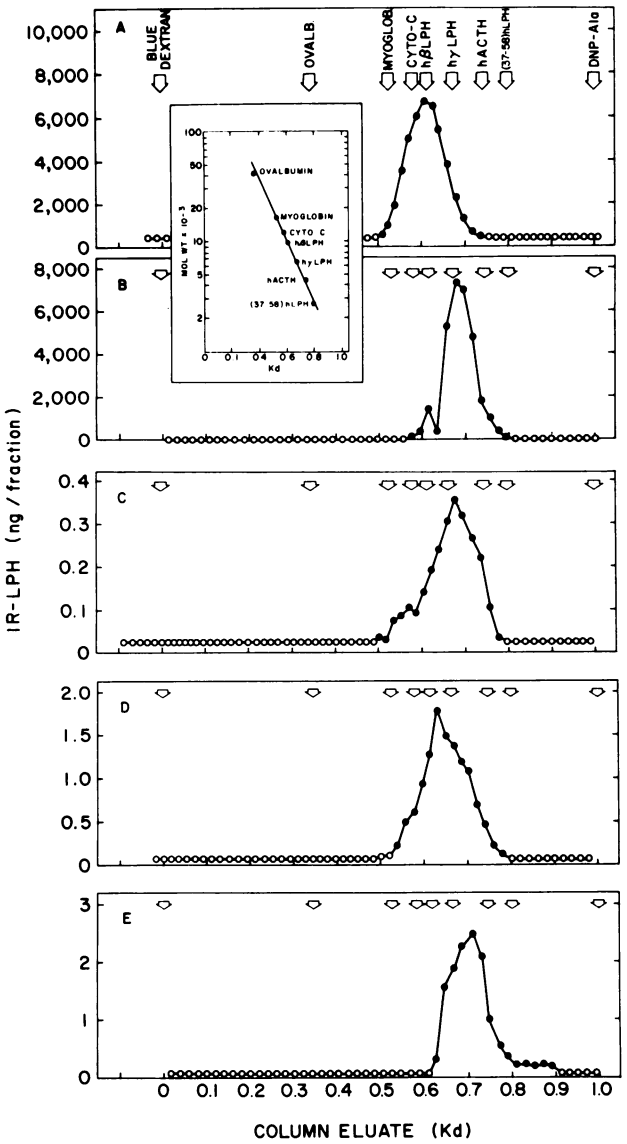

FIGURE 4 Elution profiles of pituitary and plasma IR-LPH peaks from Sepharose CL-6B in 6M Gdn/HCl. (A) The first peak (K_d = 0.21–0.33) of pituitary IR-LPH that eluted from G-75 (Fig. 1C). (B) The second G-75 peak (K_d = 0.42–0.53). (C) Sephadex G-50 void volume IR-LPH (K_d = 0.08–0.05) of a Nelson's syndrome plasma (Fig. 2B). (D) The second plasma IR-LPH peak (K_d = 0.13–0.21) (Fig. 2B). (E) The third plasma IR-LPH peak (K_d = 0.30–0.42) (Fig. 2B). Calibration with Blue Dextran 2000 (void volume), 2,4-dinitrophenyl-L-alanine (DNP-Ala) (total volume), ovalbumin, myoglobin, cytochrome *c*, purified hβLPH (Dr. Li), purified hγLPH (Dr. Lowry), and synthetic (1–39)hACTH and (37–58)hLPH is indicated at the top of each panel. The samples were incubated in column buffer containing 6 M Gdn-HCl for 18 h at 4°C before application. 19-drop fractions were collected. The insert shows the log molecular weight of the calibration standards plotted against fractional elution volume (K_d). Void volume weight was 82.49±0.83 g; total volume weight was 204.85±0.77 g.

Extraction of LPH with silicic acid. We have routinely used a modification (16) of the silicic acid extraction method of Donald (27) for LPH- (16, 23) and ACTH- (18) RIA. The usefulness of this method for extracting plasma β- and γLPH was assessed. To define the efficiency of extraction, known amounts of unlabeled or ^{125}I-labeled hβ- or hγLPH (Dr. Li and Dr. Lowry) were added to plasma obtained from normal human subjects with or without prior dexamethasone administration, respectively. The recovery of IR-LPH or ^{125}I counts per minute in the silicic acid extracts, determined in 10 experiments, was: 72.6% hβLPH, 67.8% hγLPH, 83.2% ^{125}I-hβLPH, and 93.7% ^{125}I-hγLPH, comparable to that of 78.7% for synthetic (37–58)hLPH (16).

The possibility that βLPH might be converted to γLPH during extraction was examined by adding ^{125}I-hβLPH (Dr. Li) to normal plasma or unlabeled hβLPH to fresh blood immediately after it was drawn from a normal subject whose LPH secretion had been suppressed with dexamethasone and preparing the plasma in the routine manner (16, 18, 23). The elution profiles of ^{125}I-labeled hβLPH before and after extraction from three normal plasma specimens were identical, eluting as a single peptide (K_d = 0.19). The same was true of unlabeled hβLPH added to freshly drawn dexamethasone-suppressed blood that was processed, extracted, and measured by LPH RIA in the usual manner.

These results demonstrated that the silicic acid extraction procedure was efficient for β- and γLPH and did not artifactually produce γ- from βLPH.

IR-LPH in normal plasma. Because LPH RIA sensitivity was such that small concentrations of IR-LPH peptides could easily have escaped detection in G-50 fractions of unextracted plasma, 250 ml of plasma was obtained from two normal subjects at about 8:00 a.m., extracted with silicic acid using the usual proportion of silicic acid:plasma volume (23), lyophilized, reconstituted in column buffer, and applied to the large G-50 column. In both, three peaks of IR-LPH were observed; one in the void volume, a second coeluting (K_d = 0.19) with hβLPH, and a third predominant peak coeluting (K_d = 0.37) with hγLPH (Fig. 5). The void volume competitive binding curves were again less steep than that of the standard. However, neither sample contained >0.06% of the void volume IR-LPH in an equal amount of plasma from one patient with Nelson's syndrome (Fig. 2B).

These data excluded the presence of even very small quantities of IR-LPH other than β-, γ- and void volume IR-LPH in normal plasma.

Void volume IR-LPH in plasma of patients with Nelson's syndrome. Three approaches were used to define the nature of the void volume IR-LPH in the

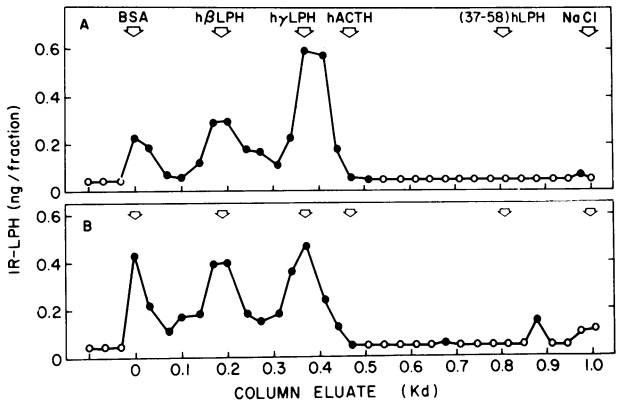

FIGURE 5 Gel filtration of plasma of two normal subjects drawn under basal fasting conditions. About 600 ml of blood was drawn at 8 a.m., plasma was prepared, and 250 ml were extracted with 100 mg of silicic acid per milliliter of plasma. The extracts were lyophilized, reconstituted in 10 ml of column buffer, and applied to the large Sephadex G-50 column. Conditions were the same as those described in Fig. 1.

plasma shown in Fig. 2B. With Sephadex G-100 gel filtration, the large IR-LPH still eluted in the void volume, indicating a molecular weight in the globulin range.

Next, the ability of silicic acid to extract void volume IR-LPH was examined. Unextracted plasma, the silicic acid extract, and the supernatant plasma left after silicic acid extraction were applied separately to the small Sephadex G-50 column (Fig. 6). Most of the

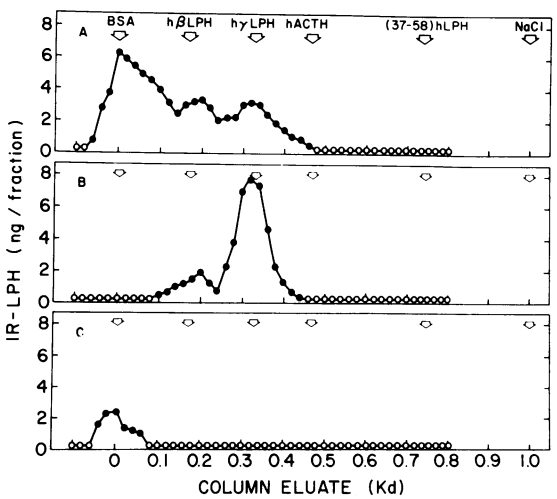

FIGURE 6 Silicic acid extraction of plasma from the patient with Nelson's syndrome (Fig. 2B) and elution from the small Sephadex G-50 column. (A) Plasma before extraction. (B) Silicic acid extract. (C) Supernatant plasma after silicic acid extraction. Conditions and data plotted as in Fig. 1, except that sample volume was 2.0 ml; fraction volume, 1.6 ml; void volume, 75.2 ml; and total volume, 155.2 ml.

void volume IR-LPH was extracted, but the supernate still contained some void volume IR-LPH (Fig. 6C). However, none of the IR-LPH extracted by silicic acid eluted in the void volume, most of the βLPH was recovered in the extract, and much more γLPH was present in the extract than in the original plasma (Fig. 6A,B). These data and those from the Gdn-HCl experiment (Fig. 4C) suggested that void volume IR-LPH was γLPH noncovalently bound to a high molecular weight protein in the patient's plasma.

This patient had not been exposed to exogenous natural porcine ACTH (contaminated with βMSH and LPH) for at least 15 yr. However, to explore the possibility that the binding protein was an antibody, freshly repurified ^{125}I-hγLPH (Dr. Lowry) was added to 0.3 ml of the patient's plasma, diluted to 2.5 ml with RIA standard diluent, and incubated for 48 h at 4°C. Plasma from a patient with Addison's disease (Fig. 2F) served as a control. Total protein-bound ^{125}I-hγLPH counts were determined by precipitation with polyethylene glycol (22). Counts bound to immunoglobulins were determined by immunoprecipitation with polyvalent and monospecific antisera to human immunoglobulins. An equal amount of ^{125}I-hγLPH in the RIA standard diluent served as a precipitation blank. The total proteins in the plasma of the patient with Nelson's syndrome bound 5.2 and 10.0% of the added ^{125}I-hγLPH in two separate experiments; the control patient's plasma with Addison's disease bound only 0.0 and 0.4%. As shown in Fig. 7, most of the protein-bound ^{125}I-hγLPH was bound to immunoglobulins, mostly to IgG, as were the small number of counts in the plasma of the patient with Addison's disease.

Thus, it appeared that the major binding protein in this patient's plasma was an antibody that reacted both with her own γLPH and with ^{125}I-labeled hγLPH added to her plasma.

DISCUSSION

The elegant studies of Scott and Lowry (5) first challenged the existence of a 22 amino acid pituitary polypeptide called "human βMSH" (4). Early studies (21) indicated that the pigmentary factor in human plasma was different from ACTH, but Abe et al. (1), using an αMSH RIA, showed that αMSH accounted for little if any of the biologic MSH activity of human plasma and tissues. However, using an hβMSH RIA, the same workers concluded that "most of the biologic MSH activity of the plasma and tissues could be accounted for by β-MSH" (2) and, thus, that "all of the available data support the view that β-MSH is the principal melanocyte-stimulating factor in human plasma" (3). The more recent studies of Scott and Lowry (5) and Bloomfield et al. (6) obviously de-

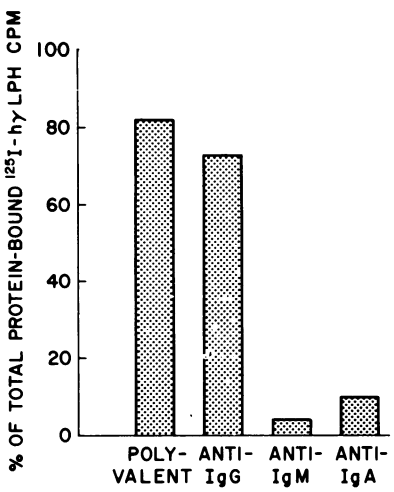

FIGURE 7 Immunoprecipitation of ^{125}I-labeled hγLPH added to plasma. The plasma of the patients with Nelson's syndrome (Fig. 2B) was incubated with ^{125}I-hγLPH (Dr. Lowry) after which the total protein-bound ^{125}I counts per minute were determined by precipitation with polyethylene glycol. The percent of the total protein-bound counts per bound by immunoglobulins was determined by immunoprecipitation with optimal concentrations of polyvalent antiserum (anti-IgG, IgM, and IgA) and monospecific antisera to human IgG, IgM, and IgA. The data are plotted as percent of the total protein-bound ^{125}I counts per minute precipitated with polyethylene glycol (5.2% of the ^{125}I-hγLPH counts per minute added to the incubation mixture in this experiment).

manded a complete reappraisal of that conclusion. As the result of our reappraisal over the past 3 yr (9–11 and the present study), we concur that hβMSH does not normally exist in human plasma or pituitary tissue, a conclusion recently reached also by Bachelot et al. (12). However, we have first demonstrated the absence of hβMSH in plasma of normal human subjects under basal conditions in the present study and have shown that the higher molecular weight IR-LPH in human plasma does not result from aggregation of hβMSH or its binding to protein. It does appear that hβMSH or a molecule similar in size may occasionally be produced ectopically by nonpituitary tumors, presumably by cleavage of β- and(or) γLPH by endopeptidases not present in normal human adenohypophysial ACTH/MSH (or, more properly, ACTH/LPH) cells (28). The present study further suggests that this ectopic tumor peptide may circulate in plasma (Fig. 3D). These tumors may also contain other melanocyte-stimulating peptides (29), apparently NH_2-terminal 1–13 to 1–15 ACTH fragments produced by tumor endopeptidases from ectopic ACTH (30).

What, then, of the "hβMSH" of the studies of Abe et al. (2, 3) and others? Because hβLPH and hγLPH contain the hβMSH sequence, antisera produced with βMSH (human or porcine) might react with the common (37–58 or 47–58, respectively) portion of the human LPH sequence. Furthermore, LPH would compete with labeled synthetic hβMSH ([37–58]hLPH) for binding to such antibodies and might generate competitive binding curves parallel to (37–58)hLPH standard (2, 6, 12, 16, 23, 31). The G-82 antiserum of Abe et al. (2, 3) cross-reacts with both γ- and βLPH (23, data kindly provided by Dr. G. A. Bloomfield and Dr. L. H. Rees), and that of Donald and Toth (32) reacts mostly with γLPH (31). Thus, by substituting LPH for βMSH, most previous studies are consistent with current knowledge about IR-LPH; repeating the studies of Abe et al. (2, 3) and others is unnecessary.

However, the use of LPH, rather than β- or γLPH, is important, because it is not yet clear which of the LPHs circulates in plasma or whether their ratio may change predictably under certain circumstances. More β- than γLPH may be released in response to hypoglycemia, for example (31), more γLPH may be produced by the pituitary, and relatively more βLPH may be produced ectopically by nonpituitary tumors (33); antisera with differential affinities for hβ- and hγLPH were used in reaching these conclusions. Bachelot et al. (12), using an antiserum raised to synthetic (37–58)hLPH and Sephadex G-50 gel filtration, found only β- and void volume IR-LPH in a pituitary extract and plasma from patients with elevated IR-LPH levels due to Cushing's disease, Nelson's syndrome, and Addison's disease, and from normal subjects after the administration of metyrapone; no γLPH was detected in any of their specimens. Although our results conflict with those of Bachelot et al. (12), our conclusion that both peptides coexist in plasma in variable relative concentrations agrees completely with those reached by the group in London (5, 6, 31, 33), and our more direct evidence (Figs. 2 and 3) also supports their observations regarding pituitary vs. ectopic LPH production (33).

The most likely explanation for the failure of Bachelot et al. (12) to find γLPH is the inaccurate calibration of their columns. We initially reached similar conclusions (9–11) because we lacked reference hβ- and hγLPH and used RNase-A (bovine pancreas, mol wt 13,700, Pharmacia Fine Chemicals, Div. of Pharmacia Inc.) to calibrate that region of the G-50 column. Use of 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis was not sufficiently precise in this molecular weight range to reveal the error (11); only when we obtained highly purified hβ- and hγLPH from Dr. Lowry and slightly more highly purified hβLPH from Dr. Li were we able to determine the identities of the IR-LPH we were studying. It is surprising that Bachelot et al. (12), who also used Dr. Li's hβLPH for calibration, failed to make this distinction. It is also possible their antiserum does not react with hγLPH, since this was not assessed (12). Finally, it is possible that βLPH was not recovered

from their columns, and the remaining γLPH peak was improperly identified. We have found that βLPH is extensively bound by glass, siliconized glass, and polypropylene in the absence of carrier protein. In our experiments with unextracted plasma, extracts of 250 ml of plasma, and tissue extracts, there was sufficient protein in the βLPH fractions (determined by OD_{280}) to result in 80–90% recovery of βLPH. However, in buffer alone or in extracts of small volumes (2–3 ml) of plasma, up to 60% βLPH was lost, not on the column, but on the walls of the fraction collection tubes. This was avoided by prior addition to the tubes of a small volume of RIA buffer with twice the usual protein concentration. In contrast, only about 15% of γLPH adsorbed to the tubes in the absence of protein.

Obviously, it is also possible that our results and those of the London group (5, 6, 31, 33) are incorrect, but this seems unlikely, since each group has reached similar conclusions using different gels, different antibodies, and different experimental methods. Our columns were calibrated with highly purified LPH preparations obtained from two independent laboratories and the results were entirely consistent. The possibility that γLPH was artifactually produced from βLPH while separating plasma from blood, extracting IR-LPH with silicic acid, eluting samples from gel filtration columns, or performing the RIA has been carefully examined; highly purified hγ- and hβLPH retained their separate identities throughout all of these procedures. The possibility that γLPH was an artifact of aggregation or protein-binding of hβMSH was also excluded. Even after correcting for the greater affinity of the G-106 and R-3 antibodies for hγ- than for hβLPH, the molar concentration of γLPH in many of the specimens far exceeds that of βLPH. Thus, we conclude that both β and γLPH normally are present in human plasma and tissue and that, in some circumstances, γLPH may predominate to the point of being essentially the only IR-LPH present.

We have demonstrated that the void volume IR-LPH in the plasma of one patient with Nelson's syndrome was predominantly γLPH bound to IgG. It is unclear why she had antibodies to γLPH. She had not received commercial porcine ACTH since the evaluation of her Cushing's disease in 1959, when she was treated with total bilateral adrenalectomy. Hyperpigmentation was first noticed 4 yr later, and she had proton beam pituitary irradiation in 1967 followed by one transfrontal and two transsphenoidal hypophysectomies over the ensuing years in unsuccessful attempts to reduce her severe hyperpigmentation. She may have been immunized by exogenous ACTH or by altered immune responses secondary to pituitary irradiation or surgery, but it appears that endogenous γLPH provided the continuing antigenic stimulus. Antibodies have been demonstrated in patients receiving exogenous ACTH (34) or other peptide hormones. Thus, caution should be exercised in interpreting the nature of "big" hormones in the plasma of patients who may have been exposed to exogenous antigens, even in the distant past, or who may have been autoimmunized as the result of their disease process or its therapy.

There is no doubt that there is a big LPH molecule, although it is as yet uncertain if it circulates in plasma. A wealth of new information about ACTH/LPH/MSH/endorphin biosynthesis has recently been developed in several laboratories, notably those of Dr. Lowry in London, Dr. E. Herbert in Eugene, Oregon, Dr. B. A. Eipper and Dr. R. E. Mains in Denver and Dr. S. Numa and Dr. S. Nakanishi in Kyoto; they cannot adequately be covered here, and even recent reviews (35, 36) are already out of date. Current evidence indicates that the ACTH/LPH/MSH/endorphin peptides are all synthesized as a common precursor molecule (mol wt 28,500–35,000), with ACTH in a roughly central position and βLPH forming the COOH-terminus (37–39). This precursor is glycosylated, increasing its apparent molecular weight (40). This glycopeptide prohormone appears to be processed differently in the anterior than in the intermediate lobe of the pituitary gland. An extension of the hypothetical biosynthetic scheme of Scott and Lowry (5) is shown diagrammatically in Fig. 8. Neither αMSH, corticotropin-like intermediate lobe peptide (41), nor βMSH appear to be produced in adult man, who has no intermediate lobe, although they may be produced during human fetal development (42). We have recently shown (43) that human

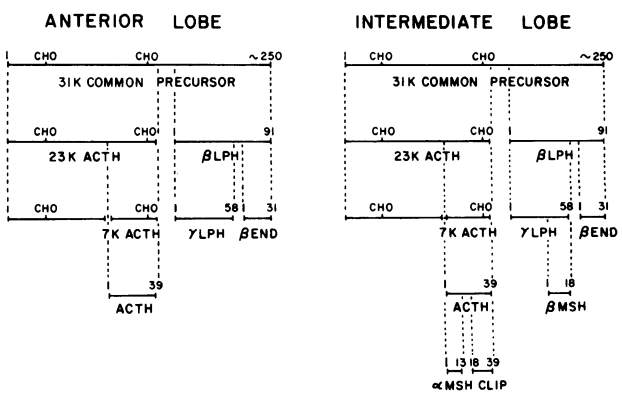

FIGURE 8 Schematic representation of postulated biosynthetic processing of ACTH/LPH/MSH/endorphin precursor. The 31,000 mol wt (31K) common precursor is converted via 23K ACTH and "intermediate" 7K ACTH to 4.5K (1–39)ACTH, βLPH, γLPH, and β-endorphin (βEnd) in the anterior lobe of the pituitary of all mammals and to αMSH, corticotropin-like intermediate lobe peptide (CLIP), βMSH, and βEnd in the intermediate lobe of the pituitary of animals that, unlike man and other primates, have one. Carbohydrate side-chains are indicated by −CHO; αMSH is *N*-α-acetyl-(1–13)αACTH-(13)amide; CLIP is (18–39)ACTH.

"big" ACTH is a glycopeptide, presumably the analogue of the 31,000 mol wt common precursor of ACTH and the LPH in the mouse (37–40). The LPH antibodies appear to react only about 10% as well with the human prohormone molecule as do ACTH antibodies (reference 44 and unpublished studies); and <1% as well with highly purified mous AtT-20/D-16v tumor 31,000 and 23,000 mol wt ACTHs provided by Dr. B. A. Eipper and Dr. R. E. Mains.[3] Thus, the sequence recognized by these antibodies may be partially obscured in the precursor molecules.

This study has three major implications. First, because LPHs apear to be only 0.2–0.4 times as potent an MSH as ACTH on a molar basis (9, 16), whereas synthetic "hβMSH" is about 20 times as potent as ACTH (16), and because LPHs circulate in concentrations very similar to those of ACTH under most conditions (2, 3, 33, 45), ACTH may yet prove to be the principal pigmentary hormone in man. Second, because LPHs are also only weak lipolytic agents, have little steroidogenic activity, and have no morphinomimetic activity (46), their exact physiological role is still in doubt. Third, the fact that γ-, not βLPH, is frequently the predominant form in plasma means that the endogenous opioid peptides, enkephalin and the endorphins (13–15), may be cleaved from the βLPH precursor (46). The synthesis of four opiate peptides by mouse AtT-20/D-16v cells in tissue culture has recently been reported (47), and we have found IR-α- and β-endorphin in an extract of a human nonpituitary tumor (44). Thus, the endorphins may be released into the blood in parallel with ACTH and γLPH under physiological situations, such as the response to stress, or under pathological conditions, such as Cushing's disease or the ectopic ACTH syndrome. They may, therefore, account for some of the psychological and physical manifestations of these normal responses and abnormal disease states.

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