The potent stimulatory effect of CRF on the secretion of pars intermedia peptides—mainly α-MSH and β-endorphin—could have major implications for our understanding of the physiological response to stress. It may indicate a role of α-MSH or circulating β-endorphin (*8*). Like thyrotropin-releasing hormone, which stimulates the release of thyrotropin and prolactin, and like somatostatin, which inhibits the secretion of growth hormone, prolactin, and thyrotropin (*9*), CRF is a hypothalamic releasing peptide with multiple sites of action.

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Rat Pro-Opiomelanocortin Contains Sulfate

Abstract. *Intermediate lobes isolated from rat pituitary glands incorporated [^{35}S]sulfate into pro-opiomelanocortin and other adrenocorticotropic hormone–containing peptides. Incubation of intermediate lobes in medium containing the arginine analog canavanine inhibited the cleavage of pro-opiomelanocortin into smaller products. Pro-opiomelanocortin that accumulated in the presence of canavanine was also sulfated.*

In recent studies sulfate has been detected covalently linked to oligosaccharides on alpha and beta subunits of the pituitary glycoprotein hormones lutropin and thyrotropin (*1, 2*) but not on human chorionic gonadotropin. The amino acid sequences of pituitary and placental alpha subunits are the same within a species. This raises the question of whether sulfation of lutropin and thyrotropin occurs as the result of an unusual capacity of pituitary cells for processing oligosaccharides or because these structurally related hormones (*3*) contain unique recognition sequences for sulfation. We sought to determine whether the pituitary glycoprotein pro-opiomelanocortin (POMC) (*4, 5*), which bears no structural relation to the thyrotropin-gonadotropin family and is synthesized in cells other than gonadotrophs and thyrotrophs, contains sulfate.

Metabolic labeling of POMC with [^{35}S]sulfate was examined in the intermediate lobe of the rat pituitary, where POMC is the major product, accounting for up to 30 percent of total protein synthesis (*4*). When intermediate lobes were incubated for 5 hours with [^{35}S]methionine or [^{35}S]sulfate, several labeled products were precipitated by antiserum to adrenocorticotropic hormone (ACTH) (lanes 1 and 3 in Fig. 1) but not by nonimmune serum (lanes 2 and 4 in Fig. 1). Methionine-labeled products had apparent molecular weights by electrophoresis of 35K, 22K, 20K, 14K, and 5K (K = 1000). Based on the known mobilities of ACTH-containing peptides in the intermediate lobe (*4, 5*), we suggest that the 35K protein corresponds to POMC, the 22K and 20K products to the NH_2-terminal segment of POMC containing ACTH, the 14K fragment to glycosylated ACTH (the molecular weight estimation of this small peptide is aberrantly high due to the oligosaccharide), and the 5K fragment to nonglycosylated ACTH. The 35K, 22K, and 14K proteins were labeled with sulfate.

Pro-opiomelanocortin contains three glycosylation sites, but the one in ACTH is glycosylated in only a fraction of the POMC molecules (*4, 5*). Presumably this contributes to the electrophoretic heterogeneity of proteins that contain ACTH domains: POMC (30K and 35K), NH_2-terminal fragment (20K and 22K), and ACTH (5K and 14K). In the case of POMC, only the highest molecular weight form was sulfated (POMC could be resolved into multiple bands by electrophoresis for longer times).

Proteolysis of POMC occurs at pairs of basic amino acid residues (Arg-Lys, Lys-Arg, or Arg-Arg), as does proteolysis of precursors of many other peptide hormones, such as insulin, gastrin, glucagon, somatostatin, and parathyroid hormone (*5*). Incubation of intermediate lobes with canavanine, an arginine analog, blocked the cleavage of POMC (lanes 2 and 3 in Fig. 2A). Moreover, the mobility of POMC was decreased by canavanine, causing an increase in apparent molecular weight from 35K to 38K. We have seen similar shifts in mobility in other peptides containing amino acid analogs. Presumably, the mobility shift reflects structural changes in the protein which are attributable to the incorporated analog (*6*). It is also possible that processing of the oligosaccharide chains may be modified by the incorporated analog. Processing of proinsulin and proglucagon can be inhibited by canavanine (*7*).

Partial sequence analysis of the 38K product synthesized in the presence of canavanine was performed after labeling with [^{3}H]leucine and [^{35}S]methionine to determine whether the NH_2 terminus of POMC was altered by incorporation of canavanine (Fig. 2B). The occurrence of leucine residues at positions 3 and 11 and the absence of methionine at the NH_2 terminus confirmed that the product has the same sequence as POMC (*8*) and is devoid of its signal peptide.

In the presence of canavanine the only sulfate-labeled product precipitated by antiserum to ACTH was POMC, and its mobility was changed by canavanine in the same way as that of the methionine-labeled product (lanes 4 and 5 in Fig. 2A). These results provide further evidence that the sulfate-labeled products seen in Fig. 1 are POMC and other ACTH-containing peptides.

The sites to which sulfate is linked in the ACTH-related species have not yet been ascertained. Some forms of gastrin have sulfate linked to the phenolic group of tyrosine, and these molecules have been detected in the intermediate and posterior lobes of the pituitary (*9*). Two considerations suggest that sulfate is not linked to tyrosine in the ACTH-related species. Acid extractions of tissue before

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immunoprecipitation did not impair recovery of sulfate-labeled products, and tyrosine-linked sulfate is quite acid-labile (*10*). Second, the appearance of sulfate is correlated with the presence of carbohydrate on different ACTH-related forms. Most notably, no sulfate was added to the smallest form of ACTH (5K) whereas sulfate did label the 14K species, which is believed to consist of the same peptide plus an oligosaccharide (*5*). Attempts to confirm the identity of 14K and 5K ACTH by NH_2-terminal sequence analysis have been unsuccessful, possibly due to blocking of the NH_2 terminus by acetylation (*5*). We have observed, in bovine and rat anterior lobes, sulfate-labeled products similar in electrophoretic mobility to the ACTH-containing products of the rat intermediate lobe. However, ACTH-related species are proportionally less abundant in the anterior pituitary, and we have not yet characterized those products by immunoprecipitation.

We conclude from the results presented here that POMC and at least two other ACTH-related peptides contain sulfate. Thus, sulfation is another step in the complex processing of POMC, which is already known to involve multiple glycosylations, maturation of oligosaccharides, proteolytic cleavages, NH_2-terminal acetylations, amidation, and phosphorylation (*5, 11*).

Pro-opiomelanocortin does not yield the same end products in the anterior and intermediate pituitary (*4, 5, 12*). Characterization of sulfation of POMC in the anterior pituitary will be necessary to assess whether it has any role in the differential processing of POMC at different sites of synthesis. Furthermore, identification of the linkage of sulfate to POMC and its relation to the sulfated linkages of lutropin and thyrotropin may aid in elucidating the physiological function of sulfation and its possible role in the sorting and packaging of glycoprotein hormones synthesized in the several cell types of the pituitary.

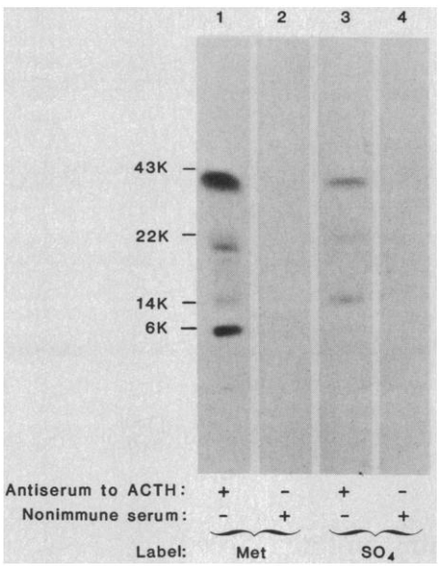

Fig. 1. Metabolic labeling of POMC with [^{35}S]methionine and [^{35}S]sulfate. Rat pituitary intermediate lobes were incubated in 0.5 ml of Krebs-Ringer bicarbonate buffer (with $MgCl_2$ substituted for $MgSO_4$) containing glucose (0.2 percent), soybean trypsin inhibitor (100 μg/ml), and penicillin G (100 U/ml) with [^{35}S]methionine (*Met*) (25 μCi/ml) (lanes 1 and 2) or [^{35}S]sulfate (SO_4) (100 μCi/ml) (lanes 3 and 4) for 6 hours at 37°C under an atmosphere of 95 percent O_2 and 5 percent CO_2. After incubation the tissue was homogenized in 1 ml of 5*N* acetic acid containing phenylmethylsulfonyl fluoride (0.6 mg/ml) and iodoacetic acid (0.6 mg/ml) (*13*) with a Ten Broeck homogenizer. The homogenate was extracted for 18 hours at 4°C and centrifuged at 10,000*g* for 30 seconds, and the supernatant was lyophilized in 0.3-ml portions. Each portion was dissolved in 500 μl of physiological saline containing 0.1 percent Triton X-100 and 5 m*M* EDTA. Seven microliters of antiserum to human ACTH linked to bovine serum albumin (provided by J.-C. Lissitsky, M. Oliver, and E. Herbert) was then added and the complex was precipitated with 25 μl of goat antiserum to rabbit serum (*8*). The precipitated proteins were resolved on 20 percent sodium dodecyl sulfate–polyacrylamide gels and detected by autofluorography. Equivalent reaction mixtures were applied to the gel. The molecular weight markers shown correspond to ovalbumin (43K), prolactin (22K), lysozyme (14K), and aprotinin (6K).

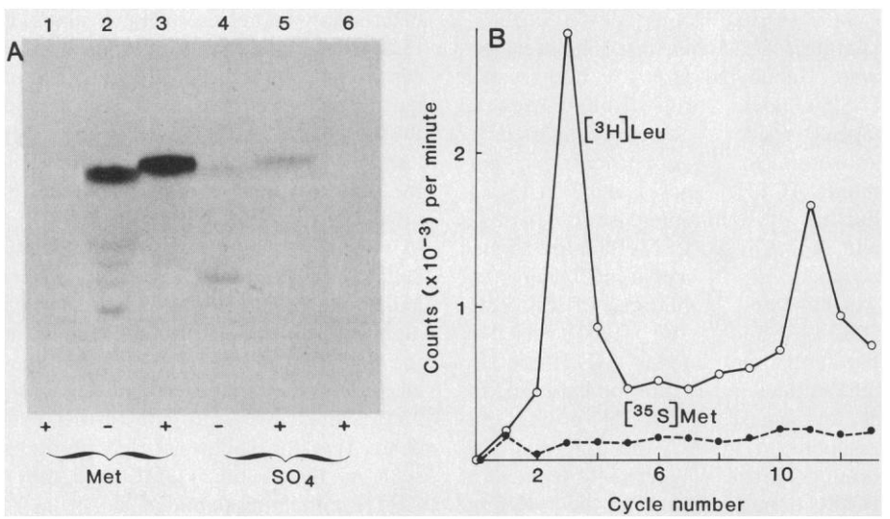

Fig. 2. Effect of canavanine on POMC synthesis. (A) Intermediate pituitary lobes were incubated in the presence (lanes 1, 3, 5, and 6) or absence (lanes 2 and 4) of 10 m*M* canavanine for 6 hours at 37°C in media containing [^{35}S]methionine (25 μCi/ml) or [^{35}S]sulfate (100 μCi/ml). The immunoprecipitated proteins were analyzed as described in the legend to Fig. 1. In lanes 1 and 6 nonimmune serum (normal rabbit serum) supplanted antiserum to ACTH. (B) Pituitaries were incubated in medium containing 10 m*M* canavanine, [^{35}S]methionine, and [^{3}H]leucine. Labeled POMC was purified by gel electrophoresis, eluted from the gel, and prepared for sequence analysis (*6*). Edman degradation was performed in a Beckman 890C sequencer with 0.33*M* Quadrol buffer. The sample applied to the sequencer contained 1.2×10^5 counts of [^{3}H]leucine per minute and 7×10^4 counts of [^{35}S]methionine per minute. Samples collected from each cycle were dried under nitrogen, dissolved in 1 ml of ethyl acetate, and counted in 1 ml of Omnifluor.

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