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THREE GROWTH HORMONE- AND TWO PROLACTIN-RELATED NOVEL PEPTIDES of M_r 13,000-18,000 IDENTIFIED IN THE ANTERIOR PITUITARY

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SUMMARY: Electrophoretic analysis of murine anterior pituitary extract revealed five major proteins of M_r 13,000-18,000 (designated P13, P14, P16, P17, and P18 according to M_r), three of which, P16, P17, and P18, were markedly influenced by estradiol benzoate and perphenazine in a manner similar to that of growth hormone, and two, P13 and P14, to that of prolactin. Tyrosine peptide mapping showed partial resemblance of fingerprints for P16 and P17 (and possibly P18) to those for growth hormone, and of P13 and P14 to those for prolactin. Both P14 and P18 bound to Concanavalin A. None of the peptides crossreacted with antibodies to growth hormone or prolactin. The concentrations of P13 and P14 in pituitaries from lactating rats and in a prolactinoma were distinctly higher than normal. All five peptides were secreted into the medium during in vitro incubation. These results suggest that P16, P17 and P18 are growth hormone- and P13 and P14 prolactin-related secretory proteins of the pituitary.

GH and PRL are the two major proteins of the anterior pituitary, which together constitute 5-10% of the total adenohypophyseal protein. Analysis of the pituitary tissue by SDS-PAGE shows five other very conspicuous bands that range in M_r from 13,000-18,000. Although these low M_r proteins can be seen in the pituitary glands of a variety of species (1, 2), their identity and chemical nature are not known. Since they are present in such large amounts in the pituitary, and since their concentrations vary among species, we examined them with physiological, structural and immunological means to determine their biological significance. Our findings suggest that these major constituents of the pituitary are secretory peptides, three of which are related to GH, and two to PRL, in regulation and structure.

Abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GH, growth hormone; PRL, prolactin; 2-ME, 2-mercaptoethanol; Con A, Concanavalin A; NTC, nitrocellulose; RIA, radioimmunoassay.

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MATERIALS AND METHODS

Animals and Tissues. S/A strain rats (Simonsen Laboratories, Gilroy, CA) and C3H/St and C57BL/6J strain mice (Scripps Clinic and Research Foundation, La Jolla, CA) were housed in controlled environment (12 hr light, 12 hr darkness; temperature 24 ± 1°C), and fed Wayne Lab Blox and water ad libitum. Anterior pituitary glands were obtained after decapitation and were washed 3-4 times with cold normal saline before being homogenized in Laemmli's sample buffer (3) in the ratio of 20 mg/ml. The homogenates were heated in boiling water for 3 min and then centrifuged at 1,000 x g for 15 min. The supernatants were stored at -20°C until analyzed by SDS-PAGE.

Physiological experiments.

(i) Effect of sex steroids. Rats and mice were assigned at random to one of the following groups: a) intact males, b) intact females, c) castrated females, d) castrated females plus estradiol benzoate (10 μg for rats, 1 μg for mice), e) intact females plus progesterone (5 mg), f) intact females plus estradiol benzoate and progesterone, g) intact females plus testosterone (500 μg). All steroids were dissolved in sesame oil. Castration was performed under light ether anesthesia. Injections were started 2 weeks after castration and were given once daily, sc, for 2 weeks.

(ii) Effect of perphenazine. Adult female rats of the S/A strain were given a single ip injections of perphenazine (1 μg/g BW) and were killed 0, 0.5, 1, and 2 hr after injection.

(iii) Effect of nursing. Primiparous rats of the S/A strain were separated from their litters for 15 hr on days 10-12 of lactation. Half of the mothers were then decapitated without nursing; the other half were allowed to nurse their young for 1 hr before decapitation.

(iv) Prolactinoma tissue. Normal and adenomatous tissues were obtained by transsphenoidal surgery from a 31 year old woman who had a long history of hyperprolactinemia and galactorrhea.

Gel electrophoresis. SDS-PAGE was performed in 1.5-mm thick and 12-cm long slab gels of 12% acrylamide using the buffer system of Laemmli (3). The gels were stained with Coomassie brilliant blue R.

Quantitation of protein bands. The amount of protein in the gel bands was determined by the method of Ball (4).

Western blotting. The method described by Burnett (5) was used. Murine proteins, electroeluted from gels, were re-electrophoresed, transferred onto NTC, and immunostained. For immunostaining, a highly specific monkey anti-murine GH serum (6) and a rabbit anti-mouse PRL serum that also crossreacts with rat PRL (7) were used at a 1:1,000 dilution. ^{125}I-labeled Protein A was used to visualize GH- and PRL-immunoreactive bands.

Peptide mapping. The method of Elder et al. (8) was used. The major peptides revealed are only those that contain tyrosine residues, but the patterns obtained are quite characteristic for a given protein.

In Vitro Incubation. Anterior pituitaries were incubated at 37°C in an atmosphere of 95% O_2 and 5% CO_2 in medium 199 (Grand Island Biological Co., Grand Island, NY), alone or in a 1:10 combination with Krebs-Ringer-Bicarbonate buffer, pH 7.4, using 0.05 ml incubation medium for each mg of tissue. At the end of either 6 or 16 hr, pituitary glands were removed, extracted as described above, and stored at -20°C for electrophoresis. The incubation medium was dialyzed against water to remove salts, lyophilized, and stored at -20°C for electrophoresis or lectin affinity chromatography.

Con A affinity chromatography. Dialyzed and lyophilized incubation medium was reconstituted in 0.01M NH_4HCO_3 and fractionated on a Con A-Sepharose 4B column, as described (9).

Bioassay. Pigeon crop sac-stimulating activity of murine P13 and P14 eluates was tested by the method described by Nicoll (10).

Statistics. Analysis of variance and Duncan's new multiple range test were used for evaluating the significance of differences among treatment means.

RESULTS

Fig. 1a,b shows the effects of sex steroids on GH, PRL, and on each of the five low M_r proteins, designated P13, P14, P16, P17 and P18, the numbers

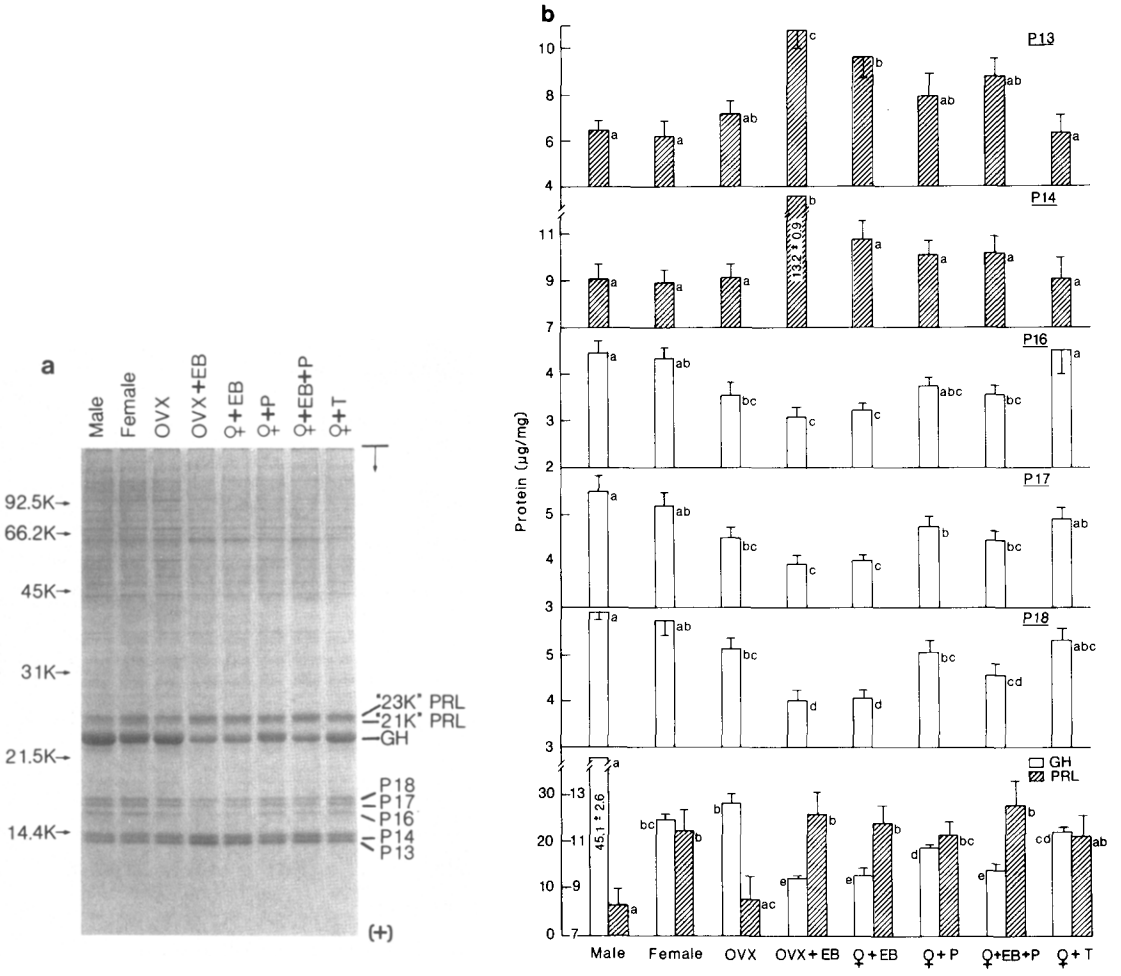

Fig. 1a. Effects of sex steroids on the low M_r proteins of the rat adenohypophysis. A 0.1 mg equivalent sample of tissue from pooled pituitary extract from each group was electrophoresed in a lane. The gel was stained with Coomassie blue. OVX = ovariectomized; EB = estradiol benzoate; P = progesterone; T = testosterone. At the time of sacrifice, the rats were approximately 90 days of age. "21K" PRL refers to a new PRL variant described recently (15).

Fig. 1b. Quantitative data for the results in Fig. 1a. Each bar is the average of 10 rats. The vertical lines represent the standard errors of the means. In the bottom panel, the outside ordinate scale pertains to GH, the inside, to PRL. The alphabetical superscripts at the top of the bars indicate statistical significance; for a given parameter, means with common superscripts are statistically not different from one another ($P > 0.05$).

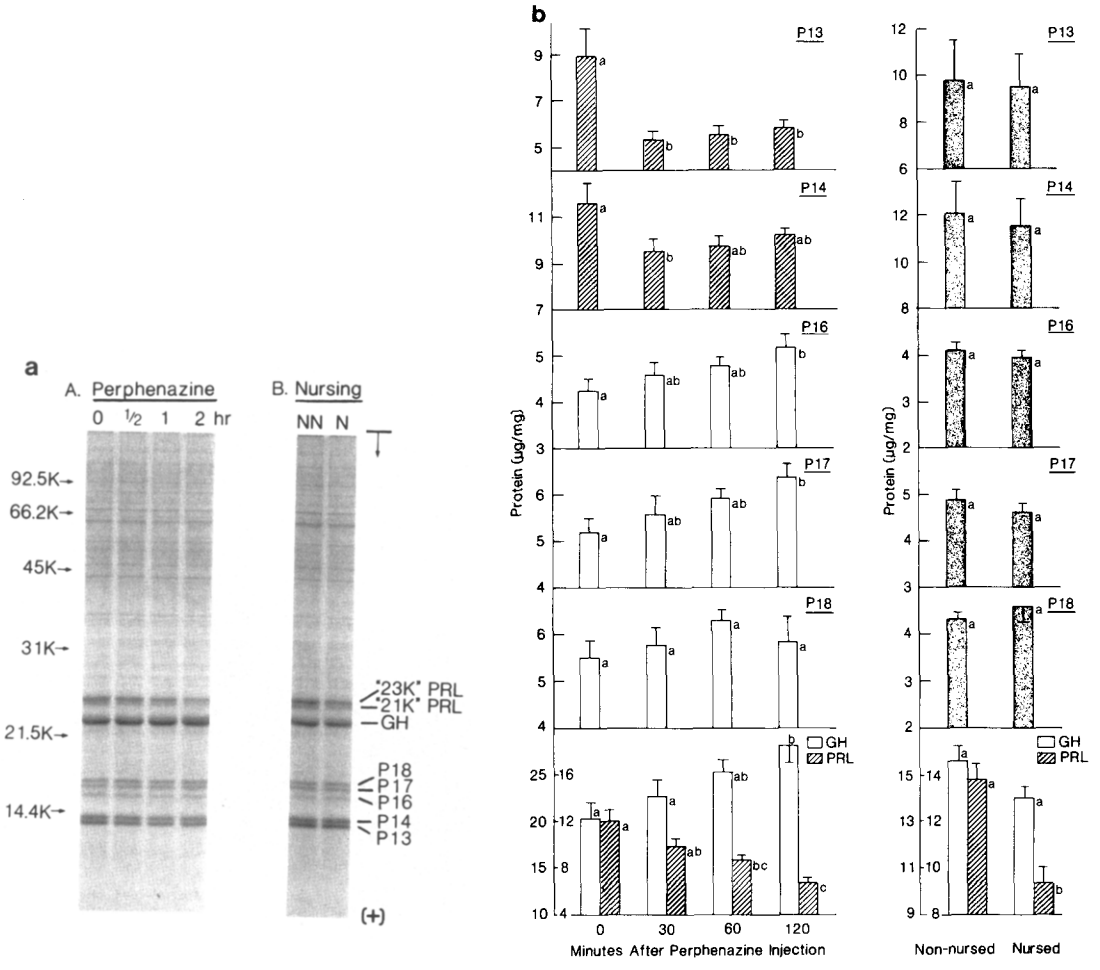

Fig. 2a. Effects of perphenazine and nursing on the low M_r proteins of the rat adenohypophysis. The rats were 108 days old at the time of the experiment. N = nursed; NN = non-nursed.

Fig. 2b. Quantitative data for the results in Fig. 2a. Each bar is the average of 8 rats. Refer to the legend of Fig. 1b for an explanation of the statistical notations.

denoting their approximate molecular mass in kilodaltons. Per mg of tissue, murine pituitary averaged 20-30 µg GH, 8-10 µg PRL, and 4-8 µg low M_r proteins. As is well known from previous work, female pituitaries contained more PRL and less GH than male pituitaries. Ovariectomy decreased the concentrations of PRL in the pituitary glands, with no sigificant changes in GH concentrations. Estradiol benzoate produced opposite effects on the two hormones: either alone or in combination with progesterone, it increased PRL concentrations but at the same time drastically reduced GH concentrations. These converse effects of estradiol benzoate were correspondingly reproduced in the case of the new proteins: the concentrations of P16, P17, and P18 were markedly reduced while those of P13 and P14 were dramatically increased. Progesterone, testosterone, or the sex of the animal had no significant effects on any of the new proteins.

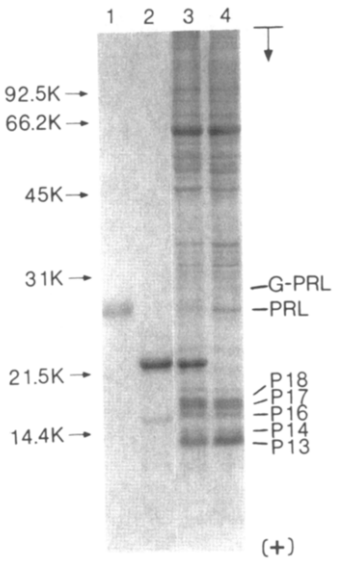

Fig. 3. Concentrations of the low M_r proteins in a human prolactinoma. Lane 1 = human PRL (Lewis 405-63-4), 25 μg; lane 2 = human GH (Lewis 201-86-3), 25 μg; lane 3 = normal pituitary tissue, 0.5 mg equivalent; lane 4 = tumorous pituitary tissue, 0.5 mg equivalent.

Fig. 2a,b shows the effects of perphenazine, a dopamine antagonist, which causes a massive release of PRL and a shut-down of GH release in the mouse (11). As expected, a single injection of the neuroleptic produced a step-wise decrease in PRL and a reciprocal increase in pituitary GH concentrations. Again, the new peptides P16 and P17 beautifully mimicked the changes observed for GH, as peptides P13 and P14 did for PRL. In contrast, acute nursing, which caused a 32% reduction in PRL concentrations and a small (9%) but insignificant drop in GH, had no appreciable effects on any of the five proteins, which speaks for the functional specificity of the new proteins. However, the concentrations of P13 and P14 were significantly greater in lactating (panel B) than in non-lactating (panel A) rats.

Fig. 3 depicts a comparison of these proteins in normal and prolactinoma tissues from a human pituitary. The prolactinoma tissue was characterized by complete absence of a GH band and a greater than normal concentration of PRL. The concentrations of P13 and P14, particularly that of P13, were also higher in the prolactinoma tissue, which indicated a common cellular origin for PRL and the two smaller peptides. On the other hand, despite the absence of a GH band, the GH-related peptides P16, P17, and P18 were present in the prolactinoma tissue, which indicated that these three peptides could be produced in non-GH-producing cells.

Fig. 4 presents the tyrosine peptide maps of the five proteins from murine and human glands and some purified preparations of GH and PRL. The fingerprint of rat P13 was similar to that of rat P14, as was the fingerprint of human P17 to human P18. Furthermore, fingerprints of the murine and human pep-

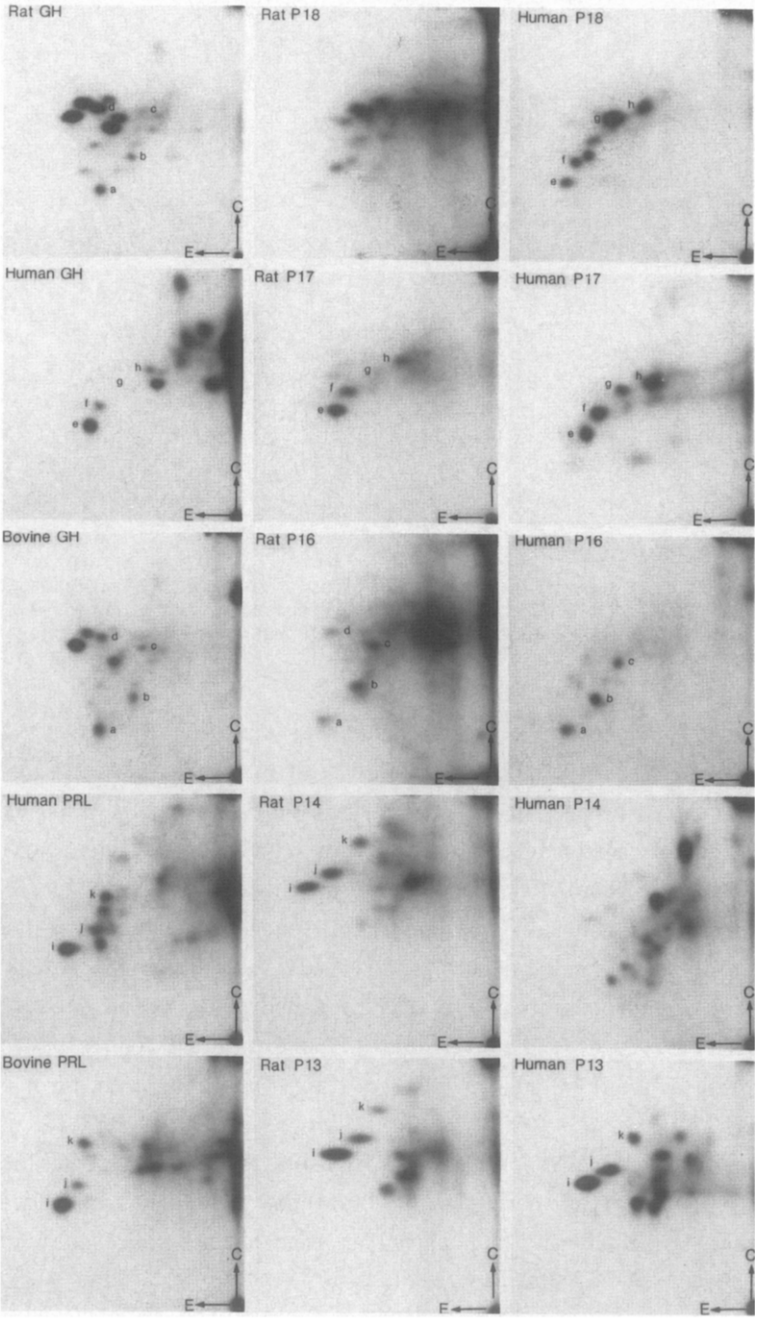

Fig. 4. Tyrosine peptide maps of the low M_r proteins from rat and human pituitaries and some GH and PRL standards. Arrows indicate the directions of electrophoresis (E) and chromatography (C).

tides exhibited easily discernible resemblances to one another in the case of three peptides, P13, P16, and P17; in the case of P14 and P18, the similarity was not so apparent. In comparison to known hormonal proteins, the migration patterns of spots a, b, c, and d of murine and human P16 exhibited striking resemblance to similar spots for bovine and murine GH. The arc-like formation

of spots e, f, g, and h of P17, in both murine and human protein, and of P18 in human protein, showed partial resemblance to a similar arrangement of spots in the human GH fingerprint. The migration patterns of spots i, j, and k of murine and human P13, and of murine P14, matched the migration patterns of similar spots in bovine and human PRL. Fingerprints of murine P18 and human P14 showed no obvious resemblance to the fingerprints of any of the purified hormones examined. Nor did the fingerprints of the new proteins resemble fingerprints of the fragments of cleaved GH or cleaved PRL (data not presented).

We then attempted to determine the immunological crossreactivity of the peptides with GH and PRL antibodies. The electroeluted murine proteins failed to crossreact with RIA-grade polyclonal antibodies to GH and PRL (data not presented). The electroeluted proteins from murine P13 and P14 bands exhibited no biological activity in the pigeon crop sac bioassay for PRL (data not presented).

Fig. 5 shows the results of *in vitro* incubation. Both GH and PRL were secreted into the medium, PRL more so than GH, in agreement with previously well-documented observations. Each of the five peptides also was present in the incubation medium, P13 in visibly greater concentration than the others. Furthermore, as we had observed in Fig. 1a, the concentrations of GH, PRL and the new peptides in the incubation medium appeared variously influenced by the sex of the animal, by ovariectomy, and by estradiol benzoate treatment.

When the incubation medium was fractionated through a Con A-Sepharose B column, P13, P15, and P17 passed unretarded, but P14 and P18 were markedly

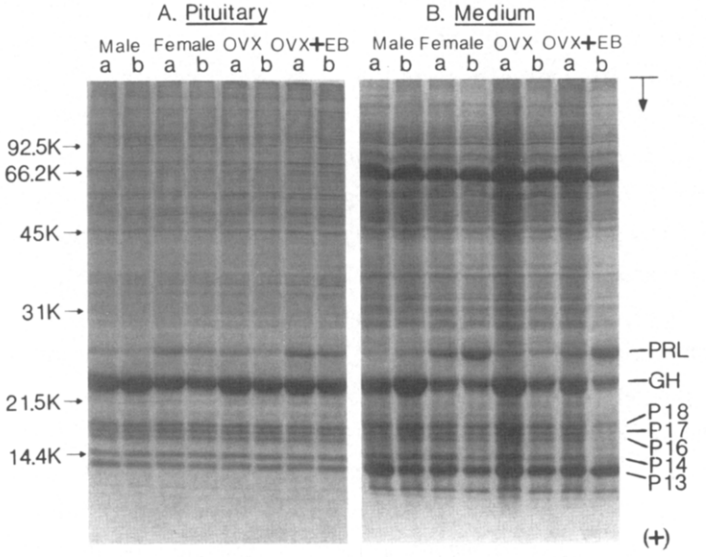

Fig. 5. *In vitro* incubation of murine pituitary for 6 hr. a = C3H/St strain; b = C57BL/6J strain. The mice were 75 days of age at the time of sacrifice. A: extract of 0.5 mg equivalent pooled pituitary tissue from each group was applied in a lane; B: medium from 10 mg anterior pituitary tissue was applied in each lane.

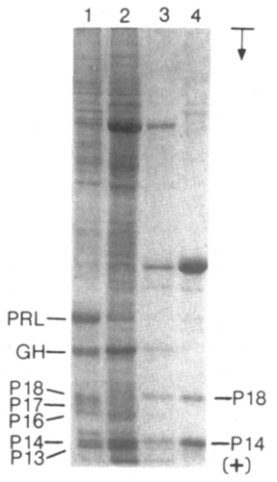

Fig. 6. Determination of the glycoprotein nature of the low M_r proteins by lectin-affinity chromatography. Medium from 16 hr incubation of 10 male rat anterior pituitaries was fractionated on a Con A-Sepharose 4B column. Lane 1 = pituitary extract; lane 2 = starting material; lane 3 = unretained fraction; lane 4 = retained fraction.

retained, and subsequently could be eluted with methyl-α-D-mannopyranoside (Fig. 6). These results indicated a glycoprotein nature for P14 and P18. Since the fingerprint of murine P14 is quite similar to that of P13, P14 may represent glycosylated P13. The same relationship could exist between P17 and P18, but the fingerprint data were not that clear-cut in this case.

DISCUSSION

Although the low M_r proteins described here are always encountered in pituitary extracts during SDS gel electrophoresis, they have remained largely unexplored. Our measurements show that they are present in the pituitary in relatively large amounts, next only to GH and PRL. Furthermore, they are subject to regulatory control, their concentrations varying under the influence of differing physiological and pharmacological stimuli. Also, they seemed to be secreted from the cell, as evidenced by their ample discharge into the medium when the pituitary was incubated *in vitro*, and by the marked drop in the concentrations of P13 and P14 when perphenazine was injected. Thus, these pituitary constituents represent not cytoskeletal proteins, but rather hitherto uncharacterized secretory peptides of the pituitary.

Induction of GH- and PRL-like changes by GH- and PRL-influencing agents, such as estradiol benzoate and perphenzine, and partial resemblance in their tyrosine peptide maps, suggest that P16, P17, and P18 are related to GH, and P13 and P14, to PRL. High concentrations of P13 and P14 in lactating rat pituitaries and in a PRL-secreting adenoma further support this conclusion. Another implicating fact is the Con A-binding property of P14 and P18, one peptide from each of the two hormone groups, which is analogous to the recently

discovered glycosylated variants of GH (12-14) and PRL (9). Whether the new peptides are fragments of GH and PRL or whether they only share common sequences with them is not clear from our results. Purification and sequencing of the peptides are needed to determine that. However, occurrence of P16, P17, and P18 in prolactinoma tissue devoid of GH and failure of acute nursing to alter P13 and P14 concentrations suggest that they are most likely produced by genes other than those for GH and PRL. The lack of immunological crossreactivity of the peptides with GH and PRL antibodies is also suggestive of this possibility.

It is not known whether the peptides have any biological action. The fact that their pituitary concentrations were altered within a relatively short time, such as only 30 min after an injection of perphenazine, suggests that they are important modulators of physiological function in the organism. Substantial increments in concentrations of the PRL-related peptides during lactation and after neoplastic transformation of the cell also imply a physiological role. The roles most likely differ from the known functions of GH and PRL, however, since the concentrations of the PRL-related peptides P13 and P14 did not change after acute nursing, despite a precipitous drop in PRL concentrations. Nor did these peptides stimulate the crop sac of the pigeon. Further work will shed light on the biological importance of these newly-recognized pituitary secretion products.

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