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Identification and Partial Characterization of a 25K Protein Structurally Similar to Prolactin[1] (42035)

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Abstract. Peptide mapping of individual pituitary proteins within the gel after separation by electrophoresis in $NaDodSO_4$-polyacrylamide gels has revealed a high-molecular-weight (mol wt) protein whose fingerprint is similar to that of prolactin (PRL). This protein is approximately 4000 greater in mol wt than the traditional pituitary PRL, but does not appear to be the latter's prohormone. Its concentration ranged from 3 to 15% of the major PRL protein in the pituitary glands of several species examined. The protein isolated from sheep pituitary glands partly cross-reacted with a polyclonal antibody raised against the main PRL, but the material from mouse pituitary glands was completely noncross-reactive. The substance eluted from denaturing gels failed to significantly stimulate mucosal growth in the crop sac of the pigeons, a commonly used test for PRL's bioactivity. Its biological activities remain to be characterized.

Plasma levels of radioimmunoassayable prolactin (PRL) often do not correlate with the biological manifestations of its secretion. For example, 86% of patients with idiopathic galactorrhea have normal levels of plasma PRL (1); murine mammary growth during pregnancy is not accompanied by a parallel rise in plasma PRL (2, 3), and a strain of mouse with larger mammary glands and high milk production has only one-half to one-third as much serum PRL as a strain with smaller mammary glands and less milk (4–7). These and other (8) observations predict the existence of PRL variants that are biologically active but nonimmunoreactive with the corresponding antisera used in current radioimmunoassays. Molecular heterogeneity characterizes several protein hormones, including human growth hormone (GH). This major pituitary hormone has a variant 2000 mol wt smaller than the usual form of the hormone (mol wt = 22,000), the so-called 20K human GH (9). Structurally, this 20K human GH lacks a chain of 15 amino acids (residues 32 to 46) from the amino terminus portion of the molecule (10) and has biological and immunological properties distinct from those of 22K human GH (9). The goal of the present study was to seek a similar molecular variant of PRL. Our attempts have uncovered several proteins having structures similar to that of the major form of pituitary PRL but different in molecular size, as indicated by their rate of migration on sodium dodecyl sulfate–polyacrylamide gels, as well as in biological and immunological activities. The subject of this communication is one of these proteins, which is approximately 3000–4000 mol wt heavier than the main PRL and can be found in as much as one-sixth of the latter's concentration in some species.

Experimental Procedures. *Animals and tissues.* Mice and rats were purchased from the Simonsen laboratory, Gilroy, California. The inbred strains such as the C3H/St and C57BL/6J mice were obtained from Scripps Clinic and Research Foundation, La Jolla, California. They were housed in air-conditioned (70 ± 1°C), light-controlled (12-hr light:12-hr dark) quarters and were given Wayne Lab Blox and tap water *ad libitum.* Their pituitary glands were removed following decapitation and the posterior lobes were separated and discarded. Rabbit, ovine, and bovine pituitary glands were purchased from Cal Med, San Francisco, California.

Gel electrophoresis. For separation of pituitary proteins by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the anterior lobes of pituitaries from all test species

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were extracted with Laemmli's (11) sample buffer (0.0625 *M* Tris–HCl, pH 6.8, 2% $NaDodSO_4$, 10% glycerol, and 0.001% bromophenol blue) with or without 5% 2-mercaptoethanol in the ratio of 20 mg/ml. The samples containing 2-mercaptoethanol were heated in a boiling water bath for 5 min. The resulting extracts were centrifuged at 10,000*g* for 10 min in a bench-top centrifuge, and the pellet was discarded. The samples were analyzed either in tube gels by using the buffer system of Weber and Osborn (12) or in slab gels with the buffer system of Laemmli (11). The tube gels consisted of 10% acrylamide and were 0.5 mm in diameter and 10 cm in length. The slab gels consisted of 12% acrylamide and were 12.5 cm long and 1.5 mm thick. Twenty-five microliters of the supernatant was applied in both cases.

Peptide mapping. For structural comparisons of proteins suspected to be related to PRL we used the peptide-mapping method of Elder *et al.* (13), which requires less than a microgram of protein. This method reveals only peptides that contain tyrosine or other iodinable residues, but the patterns are quite characteristic for a given protein. Briefly, protein bands to be mapped were excised from the gel and diced into 1- to 2-mm cubes. The gel fragments were washed exhaustively in 10% methanol to remove $NaDodSO_4$ and other contaminants. The washed fragments were dried under a heat lamp and then iodinated by sequential addition of 20 μl of 0.5 *M* sodium phosphate buffer, pH 7.5, 0.50–1.0 mCi of ^{125}I (5 μl) and 5 μg of chloramine T (5 μl). After incubation for 30 min, the reaction was stopped by adding 2 mg of sodium metabisulfite in 1 ml of water, and incubation was continued for 1 hr. The labeled gel fragments were then washed exhaustively with 10% methanol to remove free ^{125}I. Next, they were dried again in preparation for digestion of the iodinated protein, which was accomplished by adding 150 μl of a solution of TPCK-trypsin (Worthington Biochemicals, Freehold, N.J.) in 0.05 *M* ammonium bicarbonate, pH 8.0, and incubating at 37°C for 4–6 hr. The tryptic peptides were eluted by adding 0.5 ml of distilled water and incubating further for 18 hr. The supernatant containing tryptic peptides was removed and lyophilized. The lyophilized digest was dissolved in a few microliters of high-voltage electrophoresis buffer (acetic acid:formic acid: water = 15:5:80), and a 5- to 10-μl sample containing approximately 10^6 cpm was spotted on a 10 × 10-cm cellulose-coated glass TLC plate (EM Laboratories, Elmsford, N.Y.). The digest was analyzed in two dimensions by thin-layer electrophoresis in the first dimension followed by ascending chromatography in the second dimension. The chromatography buffer consisted of butanol:pyridine:acetic acid:water (32.5:25:5:20). Plates were dried at room temperature and the peptides were visualized by autoradiography on Kodak XAR film.

Immunostaining. Existence of PRL-like proteins was also probed by staining of pituitary proteins in the gel with rabbit anti-mouse or anti-bovine PRL serum by the method of Adair *et al.* (14). In some experiments, the Western blotting technique was also used (15). The anti-mouse PRL serum used is highly specific; its properties are described in (6). The anti-bovine PRL serum is also quite specific; bovine GH has <0.4% cross-reactivity with this antiserum. Lanes to be stained were cut out from slab gels and sliced transversely. Slicing allowed the antibody molecules, which could not enter into these 12% gels, to bind to proteins exposed on the surface of the gel. The sliced gels or nitrocellulose papers containing the proteins were incubated with a 1:50 or 1:100 dilution of anti-mouse PRL or a 1:10 dilution of anti-bovine PRL serum. After proper washing, the immunostained gels or papers were reacted with 2 to 3 × 10^6 cpm/ml of ^{125}I-labeled protein A (New England Nuclear, Boston, Mass.), and the reactive bands were visualized by autoradiography.

Autoradiography. The gels were dried by soaking in 70% methanol for 20 min, placing them on a thick filter paper, and applying vacuum (0.5 μm) and heat (80–82°C) for 2 hr, in a commercial slab drier (Bio-Rad, Richmond, Calif.; Model 224). Dried gels, nitrocellulose papers, or TLC plates were exposed to Kodak XAR film and a DuPont Hi-Plus intensifying screen for appropriate intervals. Film was developed in a Kodak RP X-Omat processor.

Electroelution. Proteins were recovered

from gels by electroelution. With one stained gel or lane used as a template, we cut areas of gels containing the protein bands of interest from unstained gels. These were placed in a funnel attached with a platinum wire at the rim and dialysis tubing at the stem. The gel pieces were covered with electrode buffer without $NaDodSO_4$ (5 m*M* Tris, 38 m*M* glycine, pH 8.3) and the dialysis tubing was lowered into a tray containing the same buffer. The funnel and the tray were connected to the cathodal and anodal terminals of a power supply, and electrophoresis was performed for 4 hr at 5 mA. Most of the protein eluted from the gel pieces and accumulated in the dialysis tubing by this method. Finally, the eluate was transferred into another dialysis tubing and dialyzed against distilled water to remove some of the $NaDodSO_4$ and other salts and then lyophilized.

Protein assay. The protein content of the eluates was determined by Bensadoun and Weinstein's method (16). Bovine serum albumin was used as the standard.

Bioassay. The biological activity in the eluates was determined by the pigeon crop sac bioassay for PRL, described by Nicoll (17). A 2 × 2 design with six birds per point was used. The low and high doses of the standard and unknown proteins consisted of 2 and 8 μg, respectively. Ovine PRL (NIH-P-S_{12}) with a potency of 35 IU/mg was used as the standard.

Pulse labeling. To investigate the precursor–product relationship between the new PRL variant and main PRL, the proteins were pulse labeled with radioactive amino acids *in vitro.* Anterior pituitary glands of mice were removed following decapitation, weighed, and immediately placed in a mixture consisting of Krebs-Ringer-bicarbonate buffer, Medium 199 (Gibco Laboratories, Grand Island, N.Y.) and ^{14}C-labeled amino acids (100 μCi/ml; ICN, Irvine, Calif.) in the ratio of 9:1:2.5. For each milligram of tissue, 50 μl of this mixture was used. Glands were incubated at 37°C in 95% O_2–5% CO_2 for periods up to 90 min. Afterward, further incorporation of radioactive amino acids was stopped by washing the tissue twice with chilled Medium 199 and freezing on dry ice. The frozen tissue was extracted with Laemmli's sample buffer (11) in the ratio of 20 mg/ml, and a 25-μl sample was electrophoresed. The new protein and main PRL bands were excised, solubilized in 1.0 ml of a 3:1 (vol:vol) solution of 30% hydrogen peroxide and 60% perchloric acid by heating at 75–80°C for 3 hr. We then measured radioactivity in a Beckman scintillation counter with

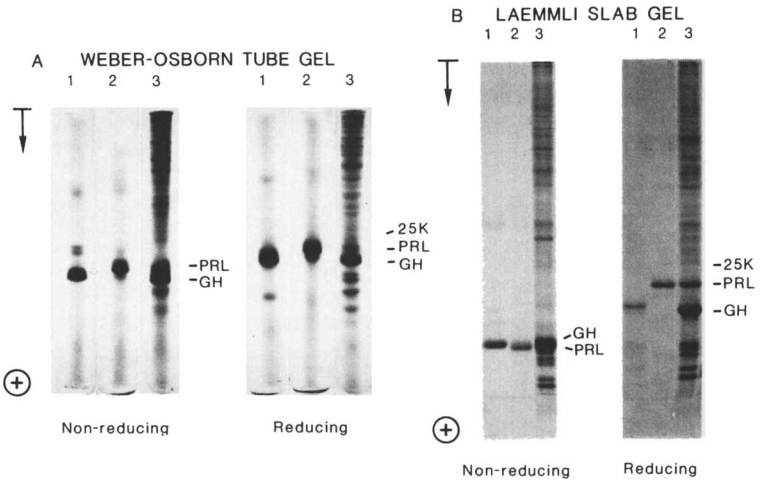

FIG. 1. Electrophoretic patterns of mouse pituitary extracts and standard mouse PRL and GH in tube gels (A) and slab gels (B) under reducing (with 2-mercaptoethanol) and nonreducing (without 2-mercaptoethanol) conditions. Lane 1: mouse GH standard, 30 μg; Lane 2: mouse PRL standard, 30 μg; Lane 3: mouse pituitary extract, 0.5-mg equivalents wet weight.

34% counting efficiency. The radioactivity in each protein band was corrected for gel background by subtracting the radioactivity found in a clear portion of the gel.

Results. Figure 1 shows the electrophoretic patterns of adenohypophyseal proteins in a crude extract of mouse pituitary analyzed in both a tube gel and a slab gel. The identities of the main PRL and GH bands were established by comparing their R_f with those of the standard mouse PRL and GH preparations. In gels without 2-mercaptoethanol (nonreducing, Fig. 1), the PRL and GH bands resolved poorly in both types of gels, because of their close mol wt. However, in the presence of 2-mercaptoethanol (reducing, Fig. 1), which reduces the disulfide bonds and unfolds the molecule, the two proteins separated, more so in the slab than in the tube gel. Furthermore, in the slab gel several minor proteins not seen in the tube gel appeared in the immediate vicinity of the main PRL and GH bands.

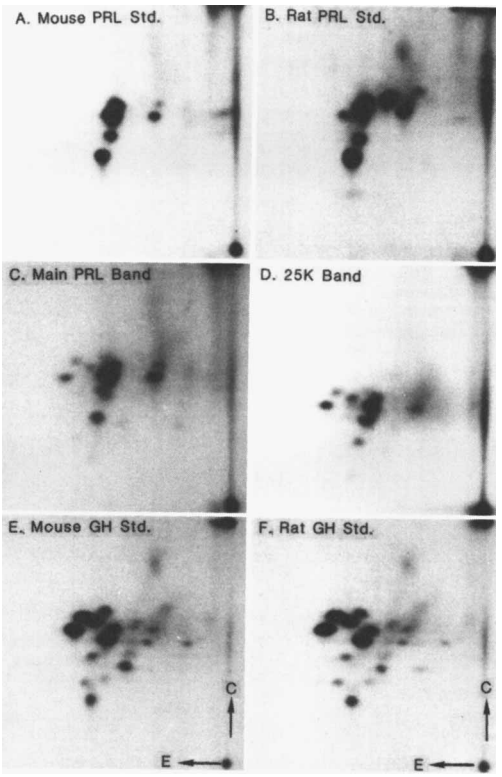

FIG. 2. Partial peptide maps of standard mouse PRL and GH and PRL and 25K bands derived from reducing gels shown in Fig. 1. Mostly tyrosine-containing peptides are visible. E, electrophoresis; C, chromatography.

FIG. 3. Diagram showing the amino acid sequence of a mouse PRL molecule based upon data published by Kohmoto *et al.* (18). One-letter symbols designate amino acids. The dark circles represent tyrosine residues. Arrows indicate the points of tryptic cleavage.

A number of these minor protein bands on either side of the main PRL and GH bands were peptide mapped. The partial peptide map of one of these newly found bands, which was more prominent than others and is tentatively designated as 25K (Fig. 1), is compared with that of the main PRL band in Fig. 2. The amino acid sequence of mouse PRL is shown in Fig. 3; it contains six tyrosine residues (18) and its tryptic digest should yield five visible peptides by the method used here. Indeed, our standard mouse PRL preparation (Fig. 2A) elicited five major spots on the TLC plate. Rat PRL contains seven tyrosine residues (19) and similar analysis of its iodinated analog should yield six spots. Standard rat PRL (NIH-P-

RP-1) (Fig. 2B) did elicit six major spots plus a few minor ones, presumably from contaminants. Mouse and rat GH would be expected to yield seven spots from the known amino acid sequence of these proteins (20, 21). Accordingly, they showed seven major and a few minor spots (Figs. 2E and F), and their fingerprints were similar to one another but distinctly different from those of PRL. As expected, the peptide map of the main mouse PRL band (Fig. 2C) was similar to that of the standard mouse PRL. But most interestingly, the fingerprint of the 25K band (Fig. 2D), which was not known to be related to PRL, appeared very similar to that of the main PRL band.

Comparative electrophoretic patterns of the 25K and main PRL proteins in the pituitary extracts of several species appear in Fig. 4. Pituitary extracts from rat, rabbit, sheep, and cow all contained the 25K band, although its concentration varied among these species. Sheep seemed to have 25K in the greatest measure, and the highest concentration of PRL as well. Densitometric analysis revealed that the 25K band ranged in concentration from 3 to 15% of the main PRL band in the various species.

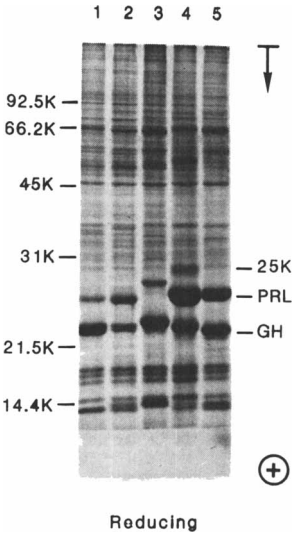

FIG. 4. Electrophoretic analysis under reducing conditions of the pituitary extracts from five species, all with the 25K protein. Lane 1: mouse, Lane 2: rat, Lane 3: rabbit, Lane 4: sheep, Lane 5: cattle. In each case, 0.5-mg equivalents of anterior pituitary tissue were electrophoresed. The positions of the mol wt markers, electrophoresed simultaneously, are shown on the left. Their mol wts are phosphorylase B, 92,500; bovine serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin, 21,500; and lysozyme, 14,400.

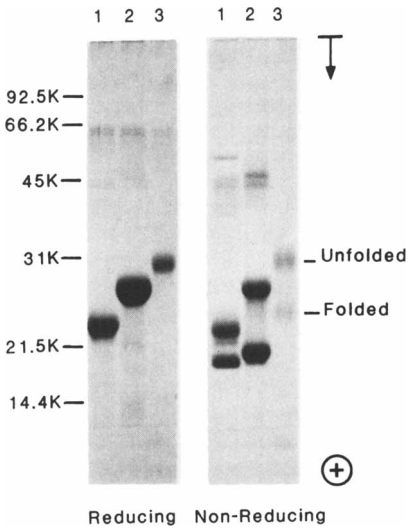

FIG. 5. Reelectrophoresis under reducing and nonreducing conditions of sheep GH, PRL, and 25K proteins eluted from reducing gels. Lane 1: eluate of GH band; Lane 2: eluate of PRL band; Lane 3: eluate of 25K band. Note that under reducing conditions each protein migrated as a single band, but under nonreducing conditions each separated into two bands, the slower band being the unfolded fraction and the faster band representing those molecules that had spontaneously oxidized and folded back. On the left side are positions of the mol wt markers described in the legend for Fig. 4.

Figure 5 shows the main PRL, 25K, and GH proteins from sheep pituitary extracts after elution from unstained, reducing gels like that in Fig. 4. The eluate then underwent dialysis, lyophilization, and reelectrophoresis in reducing and nonreducing gels. In the reducing gel, each protein migrated as expected. However, in nonreducing gel, each protein separated into two major bands. It appears that a fraction of each protein had spontaneously oxidized and folded during elution and dialysis, the folded portion migrating farther than the unfolded. A comparison of their R_f with those of mol wt marker proteins gave a value of 20,000 for the mol wt of main PRL and of 24,000 for the 25K protein in the unreduced state. Similar calculations of mol wt from reducing gels gave

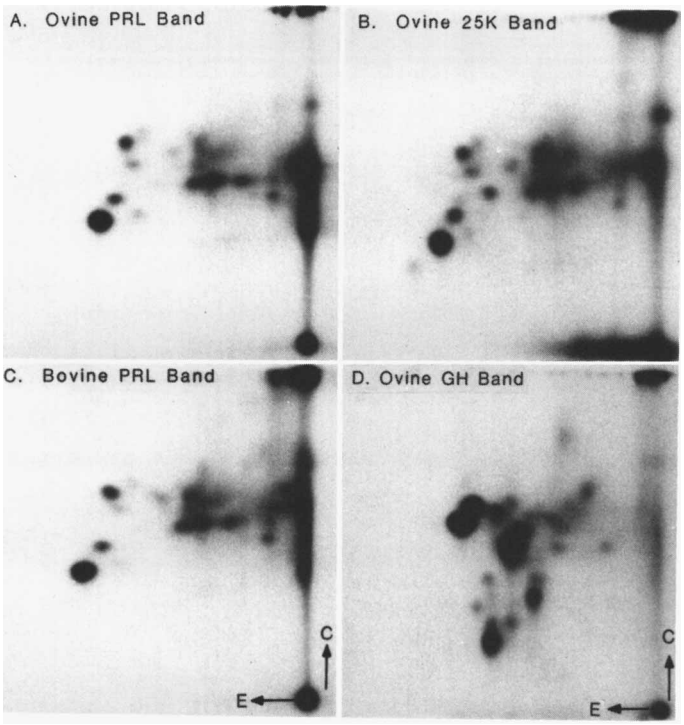

FIG. 6. Partial peptide maps of ovine and bovine pituitary proteins. For other details, see legend for Fig. 2.

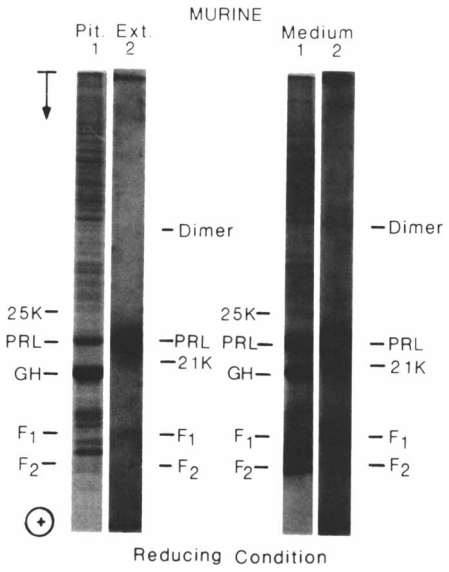

FIG. 7. Immunological cross-reactivities of mouse anterior pituitary proteins, immobilized within the gel, with antibodies against mouse PRL. A 1:50 dilution of a rabbit antiserum to mouse PRL was used for the immunostaining. Lane 1: Coomassie blue stained gel; Lane 2: autoradiograph of the immunostained gel after 48 hr of film exposure.

values that were higher: 23,000 and 28,000, respectively.

The peptide map of the ovine 25K band, depicted in Fig. 6, closely resembled the peptide maps of the main PRL band from ovine and bovine pituitaries, but was quite distinct from that of the ovine GH band.

In immunostaining experiments, the main PRL band of the mouse pituitary reacted with the PRL-antibody (Fig. 7), as did the F_1 ($\sim$16K) and F_2 ($\sim$8K) fragments of cleaved PRL (22, 23), and a high mol wt band representing, most likely, the mercaptoethanol-resistant dimer of PRL. But the 25K band of the mouse, both in pituitary extracts and incubation media, showed no appreciable cross-reactivity with the PRL antiserum. In contrast, the 25K band in ovine pituitary extracts cross-reacted strongly with antibodies

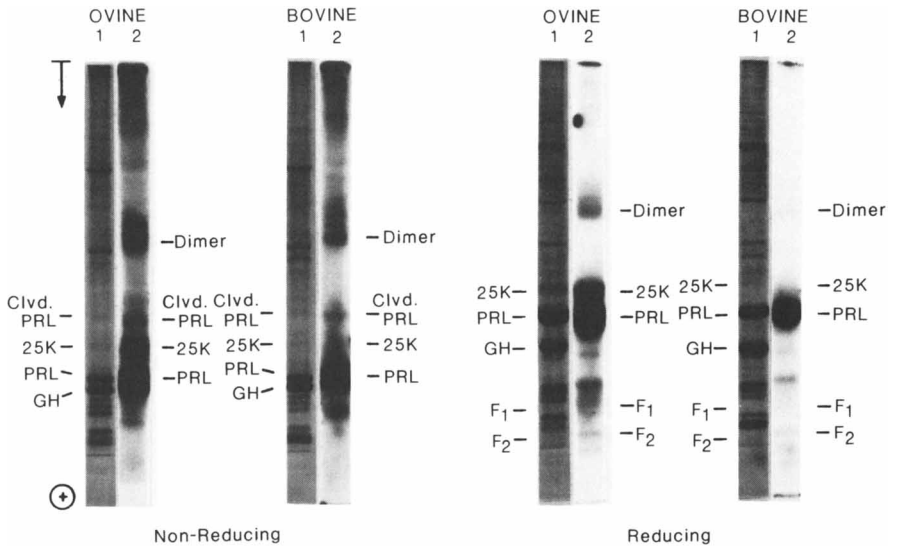

FIG. 8. Immunological cross-reactivities of ovine and bovine anterior pituitary proteins, immobilized within the gel, with antibodies raised against bovine PRL. A 1:10 dilution of a rabbit antiserum to bovine PRL was used for the immunostaining. Lane 1: Coomassie blue stained gel; Lane 2: autoradiograph of the immunostained gel after 8 hr of film exposure.

to PRL (Fig. 8), both in nonreducing and reducing gels. The 25K band from bovine pituitary extracts also cross-reacted with PRL antibodies (Fig. 8), although the reaction was much weaker in comparison to the ovine counterpart.

An unidentified band migrating ahead of the main PRL band in the mouse pituitary extract and medium, designated as 21K in Figs. 7 and 9, which we believe to be a low mol wt variant of murine PRL (to be published separately), also reacted with the PRL antibody.

Results of Western blotting experiments performed with murine pituitary extracts are shown in Fig. 9. The main PRL band of both the mouse and rat pituitary glands reacted very strongly with the PRL antibodies, as did the two fragments of cleaved PRL and a 21K band. The 25K band, on the other hand, showed no cross-reactivity whatsoever to PRL antibodies in either case.

The immunoreactivity of the 25K band was further evaluated by Western blotting experiments on its eluate. Eluates of both the main PRL and 25K bands of the mouse pituitary were reelectrophoresed in reducing

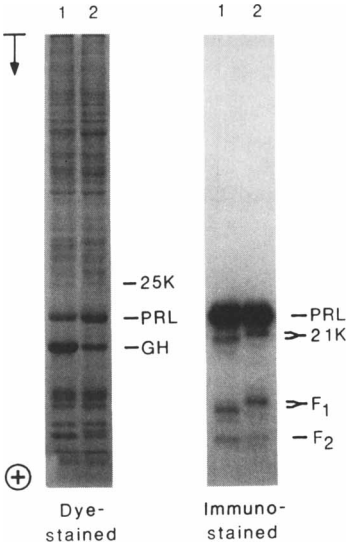

FIG. 9. Demonstration of lack of immunological cross-reactivity of murine 25K band by Western blotting. Lane 1: mouse pituitary extract; Lane 2: rat pituitary extract. In each case, 0.5-mg equivalent tissue was electrophoresed under reducing conditions, followed by transfer of the proteins onto NTC paper. The NTC paper was immunostained with 1:100 dilution of a rabbit anti-mouse PRL serum. Right panel shows autoradiograph of the immunostained NTC paper after 7 hr of film exposure.

gels and then immunostained. By dye staining, the 25K eluate showed two faint bands of contaminating proteins that migrated slightly ahead of the 25K band (Fig. 10). The R_f of the faster of the two contaminants was similar to that of the main PRL band. On immunostaining, both the contaminating proteins cross-reacted with PRL antibodies, but the 25K band did not.

The pigeon crop sac stimulating activities of the murine and ovine 25K eluates are shown in Table I. Although the standard PRL preparation and eluate of the main PRL band of ovine pituitary extracts produced marked increases in crop sac mucosal weight, eluates of the 25K protein from both murine and ovine pituitary extracts failed to do so. The eluate of the main PRL band from the mouse pituitary also failed to stimulate the pigeon crop sac significantly, but we believe this resulted from mouse PRL's inherent poor crop sac stimulating activity (Y. N. Sinha, unpublished observations). Reduction of disulfide bonds by 2-mercaptoethanol during electrophoresis and incomplete removal of $NaDodSO_4$ from the eluate may have further damaged the bioactivity. In contrast to mouse PRL, ovine PRL is highly active in the pigeon crop sac assay and, thus, is widely used as a reference preparation.

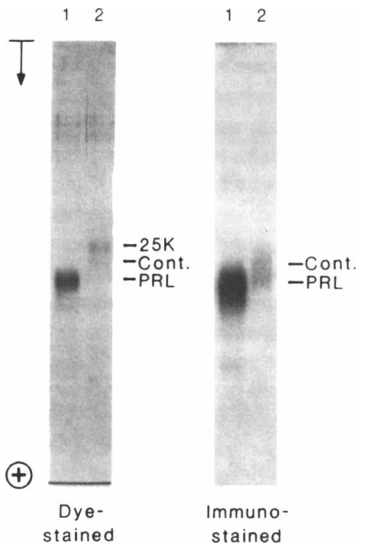

FIG. 10. Mouse 25K eluate: Western blotting of re-electrophoresed material. Electrophoresis was performed under reducing conditions. Lane 1: main PRL eluate; Lane 2: 25K eluate. Approximately 1 μg of each protein was loaded in replicate lanes. Lanes shown on the left were stained with silver; those on the right were immunostained with 1:100 dilution of a rabbit anti-mouse PRL serum. Film was exposed for 16 hr. Cont., contaminating protein.

TABLE I. PIGEON CROP SAC STIMULATING ACTIVITIES OF THE MAIN PRL AND 25K PROTEINS FROM THE MOUSE AND SHEEP ANTERIOR PITUITARY GLANDS

Species	Eluate from main PRL band	Eluate from 25K band
	IU/mg protein	
Mouse	2.6 (0.2–7.1)	4.0 (0.5–9.6)
Sheep	26.3 (7.2–67.6)	4.4 (4.8–15.2)

Note. A 2 × 2 bioassay, six birds per point. Values in parentheses indicate 95% Confidence Interval of the potency estimate. Standard used was NIH-P-S_{12}-Ovine, potency 35 IU/mg. The average index of precision (λ) for the four assays was 0.41 ± 0.05.

The kinetics of labeling of the main PRL and 25K bands in short-term incubations are shown in Table II. If the 25K protein is a precursor of the main PRL, it would be synthesized before the latter and would thus be expected to incorporate relatively greater amounts of radioactivity than the main PRL band at earlier time intervals. The amount of radioactivity incorporated in the 25K band of the mouse pituitary was lower than the main PRL band, even during very short pulses of incubation such as 1 and 2.5 min, and always constituted a very small proportion of the radioactivity incorporated in the main PRL band. This amount never exceeded the radioactivity in the main PRL band. These results indicate that the 25K protein, most likely, is not a precursor in the biosynthesis of the main PRL.

Discussion. The data presented here demonstrate the existence of a protein in the pituitary glands which could represent a new molecular variant of PRL. The identification was based upon peptide mapping of the protein by a new method (13), which allows fingerprinting of minor protein bands directly

TABLE II. TIME-COURSE OF INCORPORATION OF ^{14}C-LABELED AMINO ACIDS IN 25K AND MAIN PRL BANDS DURING SHORT-TERM INCUBATIONS OF THE MOUSE PITUITARY GLAND

Time (min)	25K band	Main PRL band	25K / main PRL ratio
	cpm/mg pituitary		
1	2 ± 2	18 ± 10	0.11
2.5	6 ± 4	58 ± 5	0.10
5	17 ± 8	550 ± 40	0.03
10	70 ± 24	766 ± 82	0.09
20	50 ± 28	1466 ± 340	0.03
45	206 ± 42	7142 ± 1222	0.03
90	616 ± 54	17142 ± 1936	0.03

Note. One mouse pituitary was incubated in each flask. Values given are means ± SEM of triplicate incubations.

from the gel. When compared to GH and PRL its peptide map was more similar to the PRL map in both species tested. This newly found pituitary protein is distinct from the 31,000 mol wt acidic protein with pigeon crop sac bioactivity but little immunoreactivity that we previously observed in mouse (24) and rat (25) pituitary glands. It is also distinct from the cleaved PRL reported by Mittra (22) and us (23) and from the preprolactin, the short-lived intermediate in the biosynthesis of PRL, reported initially by Maurer *et al.* (26). Furthermore, our pulse-labeling experiments indicate that this protein is not a prohormone of PRL.

The migration of this protein in SDS gels is slower than the main PRL band, suggesting that the molecule is larger than the standard PRL. Comparison of its relative mobility in SDS gels of 12% acrylamide with those of mol wt marker proteins indicated its mol wt to be approximately 4000 greater than the main PRL. This is a difference of nearly 20%. The structural modification responsible for this increase remains to be determined. However, recently a 25,000 mol wt form of ovine PRL has been isolated from a crude pituitary preparation, in which the increase in size was due to attachment of a carbohydrate unit at amino acid 31 of the ovine PRL molecule (27). The protein eluted from gels showed very little activity in the pigeon crop sac bioassay for PRL. This may be because nearly 60% of the material tested were in the reduced state, and it is known that cleavage of the disulfide bonds diminishes or abolishes PRL's biological activity (28). Even material purified by nondenaturing techniques from ovine pituitary glands was found to have reduced (64%) pigeon crop sac stimulating activity (27). PRL is known to have several biological actions in mammals and lower vertebrates (29); stimulation of the pigeon's crop does not constitute an action of PRL in mammals, although this highly specific response is widely used in research. Stimulation of tissues in the mammary gland, corpus luteum, prostate gland, adrenal cortex, or kidney are some of the more generic actions of this hormone in mammals, which we have not yet examined. Thus it is quite conceivable that if this 25K protein is a PRL variant, then it might have a differentiated function with respect to one or more of these target organs of PRL.

The immunological properties of the 25K protein also differed from those of the main PRL, and the difference varied between the two species examined in detail. The ovine 25K protein possessed a partial ability to cross-react with a polyclonal antibody raised against main PRL, but the murine counterpart did not do so. Therefore, in mice, and possibly other species as well, the currently available radioimmunoassay cannot measure the 25K protein; instead a specific radioimmunoassay must be developed to evaluate this protein and its potential physiological properties.

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