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Isolation, purification, and characterization of mouse placental lactogen

(placental hormone/biochemical properties/mammary gland receptor assay/immunological properties/biological activity)

PETER COLOSI, GREGORY MARR, JOANNE LOPEZ, LUIS HARO, LINDA OGREN, AND FRANK TALAMANTES*

Thimann Laboratories, University of California, Santa Cruz, California 95064

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ABSTRACT **Mouse placental lactogen was purified 1840-fold from BALB/c placentae from days 14–18 of gestation with an overall yield of 29%. The purification procedure included alkaline homogenization and extraction, ammonium sulfate precipitation, hydrophobic interaction chromatography on Phenyl-Sepharose, ion-exchange chromatography on CM- and DEAE-cellulose, and gel exclusion chromatography on Sephadex G-100. On 10% alkaline polyacrylamide gels, mouse placental lactogen had an R_f of 0.19. Electrophoresis in gels containing $NaDodSO_4$ showed a single band with a mobility corresponding to a M_r of 23,000 ± 1000. The isoelectric point, determined by isoelectric focusing in 8 M urea/5% 2-mercaptoethanol, was 7.1. When tested in the pigeon crop sac assay, 10 μg of mouse placental lactogen produced stimulation comparable with that evoked by 10 μg of ovine prolactin. In the rabbit mammary gland radioreceptor assay, mouse placental lactogen was 150% more potent than ovine prolactin in displacing ^{125}I-labeled ovine prolactin from rabbit mammary gland membranes. Iodinated purified mouse placental lactogen could be displaced from rabbit mammary gland membranes by mouse placental lactogen, mouse prolactin, and ovine prolactin. Ovine prolactin was 45% as avid as mouse placental lactogen in displacing ^{125}I-labeled mouse placental lactogen from rabbit mammary gland membranes. Mouse placental lactogen did not crossreact with antisera to mouse prolactin or mouse growth hormone in a radioimmunoassay.**

Placental lactogens (PLs) from a number of species have been purified and biochemically characterized (for review, see ref. 1). Among the rodents, rat PL has been isolated, purified, and characterized (2, 3). Although the presence of a mouse PL (mPL) is well established (4, 5), the hormone has not yet been purified to homogeneity nor have its biochemical, immunological, and receptor-binding properties been thoroughly characterized.

Apart from its use in the development of a specific homologous radioimmunoassay, a purified preparation of mPL would permit examination of its physiological role during pregnancy. In addition, the evolutionary relationship between mPL and PLs from other species could be determined once purified mPL is available.

The present study reports the isolation and purification of mPL. In addition, several biochemical, immunological, and receptor-binding properties of mPL were compared with those of mouse prolactin (mPRL) and mouse growth hormone (mGH).

MATERIALS AND METHODS

Hormone Purifications. mPRL was purified (6) and characterized (7) by procedures established in this laboratory. mGH was purified from side fractions obtained during the purification of mPRL. Fractions from Sephadex G-100 and DEAE-cellulose columns that contained mGH as shown by gel electrophoresis were pooled and dialyzed against 25 mM Tris·HCl, pH 8.5. The sample was applied to a 1 × 30 cm DEAE-cellulose column equilibrated with 25 mM Tris·HCl, pH 8.5, and eluted with a 600-ml linear salt gradient in which the limiting buffer contained 600 mM NaCl. Fractions containing mGH, which eluted at 240 mM NaCl, were concentrated by ultrafiltration through an Amicon YM-10 membrane and chromatographed on a 2.6 × 100 cm Sephadex G-100 column equilibrated with 50 mM NH_4HCO_3, pH 8.5. The mGH fraction [elution volume/void volume ratio (V_e/V_0), 1.6] was concentrated with a YM-10 membrane and lyophilized. mPL was purified by the following procedure. All steps were carried out at 6°C. Extracts, fractions from ammonium sulfate cuts, and column eluates were assayed for prolactin-like activity by a radioreceptor assay using ^{125}I-labeled ovine prolactin (oPRL) as the tracer and oPRL (NIH-PS-13) as the standard. Column eluates that did not contain Triton X-100 were monitored at 280 nm while those containing detergent were monitored at 290 nm.

Three hundred grams of frozen placentae, obtained from ≈500 BALB/c mice at days 14–18 of gestation, were thawed and rinsed in 0.9% NaCl. The tissue was homogenized in 1500 ml of 0.1 M NH_4HCO_3/NH_4OH/10 mM EDTA/10 mM EGTA/1 mM phenylmethylsulfonyl fluoride, pH 9.3, with a Polytron homogenizer for 2 min at top speed. Extraction was carried out for 12 hr with slow stirring. After extraction, the homogenate was slowly brought to pH 8.2 with 50% acetic acid and then centrifuged at 27,000 × *g* for 45 min. The supernatant was brought to 20% saturation by the slow addition of finely powdered $(NH_4)_2SO_4$. The mixture was stirred for 1 hr, and the precipitate was removed by centrifugation at 27,000 × *g* for 30 min. The supernatant was brought to 45% saturation and stirred for 1 hr, and the precipitate was collected as before. The pellet was solubilized in 215 ml of 0.1 M NH_4HCO_3/NH_4OH/10 mM EDTA/10 mM EGTA, pH 9.0, and applied to a 2.6 × 44 cm phenyl-Sepharose column equilibrated with solubilization buffer. The column was then washed with ≈3 bed vol of 5 mM sodium glycinate, pH 9.0, and eluted with 5 mM sodium glycinate/55% ethylene glycol, pH 9.0. Fractions showing radioreceptor assay activity were pooled and dialyzed first against 5 mM sodium glycinate/250 mM NaCl/0.1% Triton X-100, pH 9.0, for 2 hr and then against 20 mM sodium citrate/0.1% Triton X-100, pH 5.7, for 8 hr. The sample was then centrifuged at 27,000 × *g* for 30 min and the supernatant was applied to a 1.6 × 14 cm CM-cellulose column equilibrated with 20 mM sodium

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Abbreviations: PL, placental lactogen; mPL, mouse placental lactogen; mGH, mouse growth hormone; hPL, human placental lactogen; mPRL, mouse prolactin; oPRL, ovine prolactin; oPL, ovine placental lactogen; V_e/V_0, elution volume/void volume ratio.

* To whom reprint requests should be addressed.

citrate/0.1% Triton X-100, pH 5.7. The column was washed with 35 mM sodium citrate/0.1% Triton X-100, pH 5.7, until the absorbance at 290 nm returned to baseline and then eluted with a 420-ml linear gradient formed by mixing equal volumes of 35 mM sodium citrate/0.1% Triton X-100, pH 5.7, and 250 mM sodium citrate/0.1% Triton X-100, pH 5.7. Fractions showing radioreceptor assay activity were pooled and dialyzed against 10 mM NH_4HCO_3/NH_4OH, pH 9.0. The dialyzed material was applied to a 1.6 × 35 cm DEAE-cellulose column equilibrated with 10 mM NH_4HCO_3/NH_4OH, pH 9.0. The residual detergent was removed with 5 bed vol of starting buffer. The prolactin-like activity was eluted with a 1000-ml linear salt gradient using 290 mM NH_4HCO_3/NH_4OH, pH 9.0, as the limiting buffer. Fractions showing radioreceptor assay activity were pooled, concentrated to 5 ml by ultrafiltration through an Amicon YM-10 membrane, and applied to a 2.6 × 95 cm Sephadex G-100 column equilibrated with 30 mM NH_4HCO_3, pH 9.0. The mPL-containing fractions were pooled and lyophilized.

Electrophoresis. Discontinuous polyacrylamide gel and $NaDodSO_4$ electrophoresis on 10% gels were carried out as described (8).

Isoelectric Focusing. The isoelectric points of mPL, mPRL, and mGH were determined by using a method based on that of O'Farrell (9). The gels contained 2% Ampholyte (pH, 4.83–7.50) in the presence of 4% acrylamide/8 M urea. Sample buffer also contained 5% 2-mercaptoethanol.

Radioreceptor Assay. ^{125}I-Labeled oPRL and rabbit mammary gland membranes were prepared as described (10). mPL was iodinated by a lactoperoxidase method. Five micrograms of mPL in 10 μl of 50 mM sodium phosphate (pH 7.5), 0.5 μg of lactoperoxidase in 20 μl of 0.5 M sodium phosphate (pH 7.5), and 100 ng of freshly diluted H_2O_2 were added to 1 mCi of carrier-free $Na^{125}I$ (1 Ci = 3.7×10^{10} becquerels), and the mixture was allowed to react for 3 min and then diluted with 0.3 ml of 25 mM Tris·HCl, pH 7.5. ^{125}I-Labeled mPL was purified from the reaction mixture as described (8). The assay conditions used were identical to those reported earlier (8). Because oPRL was used as the standard in assays in which purification steps were monitored, activity was expressed as micrograms of oPRL equivalents per milliliter.

Immunological Properties. The crossreactivity of mPL against anti-mPRL was examined in a double-antibody radioimmunoassay (11). The crossreactivity of mPL against anti-mGH was examined in a double-antibody radioimmunoassay previously described (12).

Biological Activity. The prolactin-like biological activity of mPL was assessed in the pigeon crop-sac assay according to the method of Nicoll (13).

RESULTS

Hormone Purification. The purification of mPL is summarized in Table 1. Two and one-half milligrams of purified mPL were obtained from 300 g of mouse placentae, representing a recovery of 29% of the radioreceptor assay activity of the starting material. The specific activity of the mPL was 1.2 mg oPRL equivalents/mg of protein, which represented a purification of 1840-fold relative to the specific activity of the initial NH_4HCO_3 extract. The elution profile of the 20–45% $(NH_4)_2SO_4$ pellet from phenyl-Sepharose is shown in Fig. 1. mPL was relatively hydrophobic and eluted only after 55% ethylene glycol was included in the buffer. The elution pattern obtained when mPL-containing fractions from the phenyl-Sepharose column were pooled and chromatographed on CM-cellulose is shown in Fig. 2. mPL eluted between 55 and 96 mM sodium citrate/0.1% Triton X-100, pH 5.7. This step, which removed most of the hemoglobin, increased the specific activity 4-fold with 95% recovery. When cation-exchange chromatography was carried out in the absence of Triton X-100, significant loss of mPL occurred. When the mPL-containing fractions from the CM-cellulose column were chromatographed on DEAE-cellulose, the elution profile showed three peaks which absorbed at 280 nm (Fig. 3). The large plateau contained residual Triton X-100, and the third peak, which eluted between 105 and 125 mM NH_4HCO_3/NH_4OH, pH 9.0, contained mPL. Although the mPL peak was symmetrical and well separated from the other peaks, the mPL-containing fractions showed some absorbance at 410 nm. Consequently, the mPL fractions were concentrated by ultrafiltration and chromatographed on Sephadex G-100 (Fig. 4). mPL eluted at $V_e/V_0 = 2.0$. Only the peak eluting at $V_e/V_0 = 1.5$ showed absorbance at 410 nm.

Electrophoresis. Electrophoretic homogeneity of mPL, mGH, and mPRL was monitored on 10% and 10% $NaDodSO_4$ alkaline polyacrylamide gels (Fig. 5). mPL, mGH, and mPRL migrated with R_fs of 0.19, 0.21, and 0.49, respectively, on alkaline polyacrylamide gels. On $NaDodSO_4$-containing gels, mPL, mGH, and mPRL migrated as single bands of M_r 23,000 ± 1000, 21,000 ± 1000, and 23,000 ± 1000, respectively. All three hormones migrated as single bands on $NaDodSO_4$-containing gels only when the concentration of 2-mercaptoethanol was ≥10%. When lower concentrations of the reducing agent were used, additional bands of M_r 41,000 ± 1000 and 19,000 ± 1000 were observed. This phenomenon was most pronounced for mPRL. It seems likely that the M_r 41,000 band represented a disulfide dimer and the M_r 19,000 band was a monomer that was not fully reduced.

Isoelectric Focusing. The isoelectric points of mPL, mGH, and mPRL were 7.1, 7.0, and 7.1, respectively (data not shown).

Table 1. Recovery and activity of mPL throughout purification

Purification step	Protein,* mg	Activity		Yield, %	
		Total,† mg	Specific,‡ %	Successive	Cumulative
NH_4HCO_3/NH_4OH, pH 9.3, extract	16,562	10.8	0.065	100	100
$(NH_4)_2SO_4$ precipitation					
20%	14,027	9.4	0.067	87	87
45%	7,095	8.9	0.125	94	82
Chromatography					
Phenyl-Sepharose	96	5.3	5.520	60	49
CM-cellulose	22	5.0	22.720	95	46
DEAE-cellulose	10	4.0	40.000	79	37
Sephadex G-100	2.5§	3.1	120.000	78	29

* Calculated by the procedure of Bradford (14).
† Measured by radioreceptor assay using oPRL as the standard.
‡ Calculated as (mg oPRL equivalents/mg of total protein) × 100.
§ Dry weight.

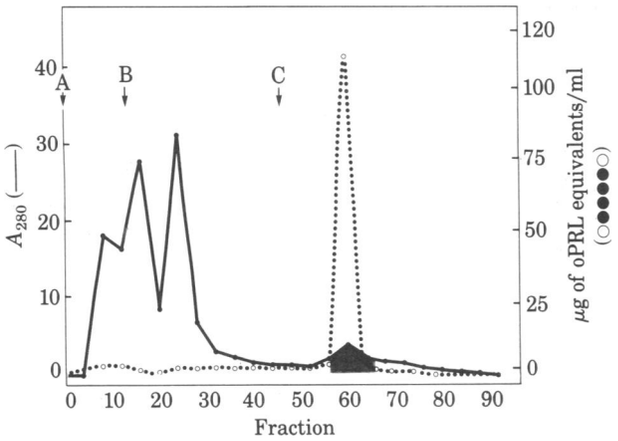

FIG. 1. Phenyl-Sepharose chromatography of 20–45% ammonium sulfate pellet. A to B, load sample; B to C, wash with 5 mM sodium glycinate, pH 9.0; C, elute with 5 mM sodium glycinate/55% ethylene glycol. Fractions were 20 ml per tube.

Radioreceptor Assay. The displacement of ^{125}I-labeled oPRL from rabbit mammary gland membranes by various concentrations of mPL, mPRL, human PL (hPL), and oPRL is shown in Fig. 6. The displacement curves for mPL, mPRL, and hPL were parallel to that of oPRL. mGH did not displace ^{125}I-labeled oPRL at concentrations as high as 10,000 ng/ml (data not shown). mPRL was 10.6%, hPL was 15%, and mPL was 150% as effective as oPRL in competing with labeled oPRL for binding sites on rabbit mammary membranes. The displacement of ^{125}I-labeled mPL by mPL, oPRL, and mPRL in the radioreceptor assay is shown in Fig. 7. The displacement curves for oPRL and mPRL were parallel to that of mPL. oPRL was 48.5% and mPRL was 3.1% as effective as mPL in competing with labeled mPL for binding sites on rabbit mammary membranes.

Immunological Properties. Amounts of mPL that ranged from 0.001 to 1000 ng did not displace ^{125}I-labeled mPRL in the mPRL radioimmunoassay. Similarly, amounts of mPL that ranged from 0.001 to 10,000 ng did not displace ^{125}I-labeled mGH in the mGH radioimmunoassay (data not shown).

Biological Activity. The prolactin-like activity of mPL was verified in the pigeon crop-sac assay. The activity of 10 μg of mPL was comparable with that of 10 μg of oPRL (NIH-PS-14; data not shown).

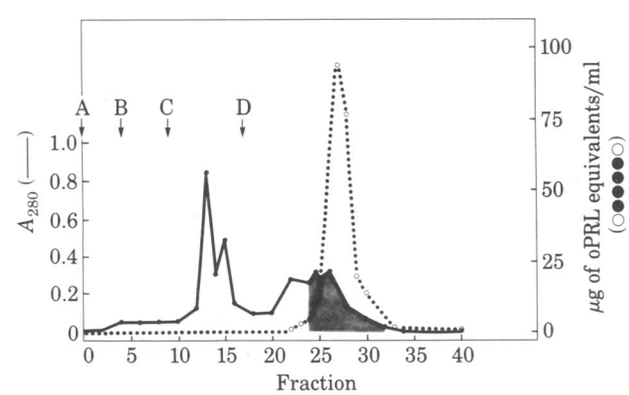

FIG. 2. Elution profile of mPL-containing fractions from the phenyl-Sepharose column on CM-cellulose. A to B, load sample; B to C, wash with 20 mM sodium citrate/0.1% Triton X-100, pH 5.7; C to D, wash with 35 mM sodium citrate/0.1% Triton X-100; D, start linear salt gradient. Fractions were 10 ml per tube.

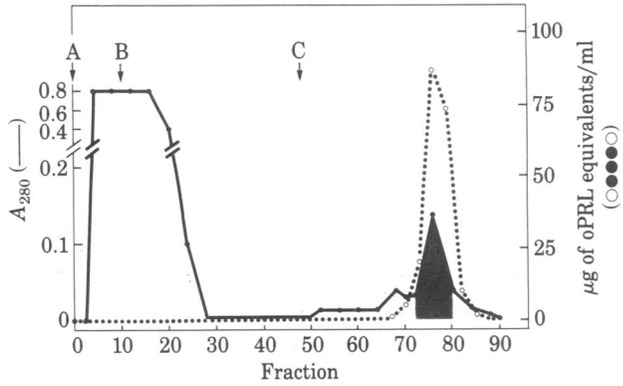

FIG. 3. DEAE-cellulose chromatography of mPL-containing fractions from the CM-cellulose column. A to B, load sample; B to C, wash with 10 mM NH_4HCO_3/NH_4OH, pH 9.0; C, start linear salt gradient. Fractions were 10 ml per tube.

DISCUSSION

This study reports the complete isolation, purification, and characterization of mPL. mPL is a single-chain polypeptide having an isoelectric point of 7.1 and a M_r of 23,000 ± 1000. The elution volume of the molecule on Sephadex G-100 (V_e/V_0 = 2.0) indicates that mPL is probably monomeric. mPL is relatively hydrophobic, as evidenced by the fact that it was strongly retarded by phenyl-Sepharose. A similar strong affinity for hydrophobic surfaces has also been shown for human prolactin and human growth hormone during hydrophobic interaction chromatography (15). We have observed that, like mPL, hPL requires a minimum of 50% ethylene glycol to be eluted from phenyl-Sepharose (data not shown).

Purified mPL was free of contamination by mGH and mPRL when analyzed by radioimmunoassay for these hormones. On a 10% alkaline gel system, mPL was resolved into a major and a minor band. Major and minor bands were also observed for mGH and mPRL. The faster migrating bands in these hormone preparations may represent nonenzymatically deamidated forms, as has been reported for other lactogens (6, 16).

Sakai and Kohmoto (5) have reported the presence of two distinct bands with radioreceptor assay activity having widely differing R_Fs in eluates of gel segments obtained after electrophoresis of crude homogenates of mouse placentae from day 16 of gestation on 10% alkaline polyacrylamide gels. The R_F of the slower migrating band was similar to the R_F of purified mPL

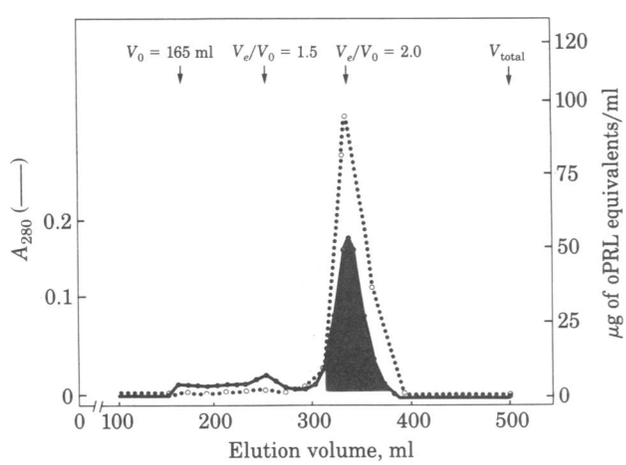

FIG. 4. Elution profile of mPL-containing fractions from the DEAE-cellulose column on Sephadex G-100.

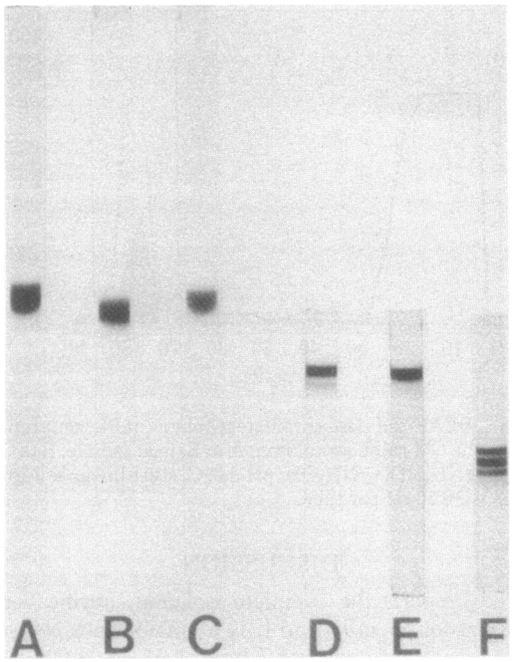

FIG. 5. Electrophoresis of mPL, mGH, and mPRL on 10% Na-DodSO$_4$/polyacrylamide gels (lanes A–C) and on 10% alkaline polyacrylamide gels (lanes D–F). The bottom of each gel indicates the distance traveled by the tracking dye. Lanes: A, mPL (12.5 μg); B, mGH (12.5 μg); C, mPRL (12.5 μg); D, mPL (10 μg); E, mGH (10 μg); F, mPRL (10 μg).

reported here. The M_r of this band was estimated to be 23,000, and the material showed considerably greater binding to mouse mammary gland slices than did material from the faster migrating band (R_F, 0.71). The nature of the binding activity and the molecular weight of the second (faster migrating) band were not investigated by these workers. At present, it is not clear what factors underlie the differences between the presence of two electrophoretically different forms in their study and the presence of only one form of purified mPL in our studies.

The M_r of 23,000 observed for mPL in our studies is comparable with values reported for PLs for the rat (3), sheep (17, 18, 19, 20), goat (21), and human (22).

The isoelectric point of 7.1 for mPL is similar to the isoelectric points of 6.8 and 7.2 reported for ovine PL (oPL) by Hurley *et al.* (19) and by Martial and Djiane (17). Bovine PL and hPL are much more acidic than mPL, having isoelectric points of 5.9 and 5.7, respectively (23, 24). The isoelectric point of rat

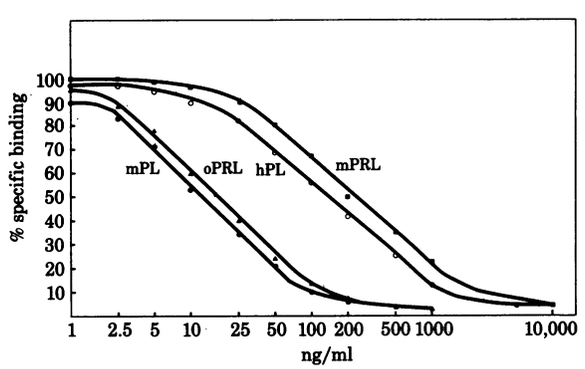

FIG. 6. Displacement of ^{125}I-labeled oPRL from rabbit mammary gland membranes by various concentrations of mPL, oPRL, hPL, and mPRL.

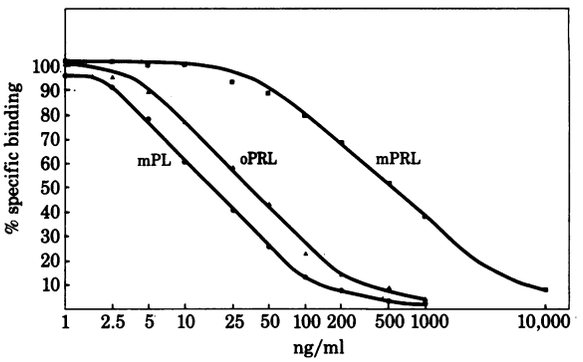

FIG. 7. Displacement of ^{125}I-labeled mPL from rabbit mammary gland membranes by various concentrations of mPL, oPRL, and mPRL.

PL can be extrapolated to be between 6 and 7 (2). The most basic isoelectric points that have been reported for PLs are 8.2–8.4 and 8.8 for oPL (20) and caprine PL (21), respectively.

mPL showed excellent prolactin-like activity with respect to its ability to displace ^{125}I-labeled oPRL from rabbit mammary gland membranes. mPL was 150% more potent than oPRL in displacing labeled oPRL, whereas mPRL was only 10.6% as potent as oPRL. Our preparation of mPL was easily iodinated and the iodinated material could be displaced by mPL, mPRL, and oPRL. It is interesting to note that, although oPL is equipotent with oPRL in the rabbit mammary gland radioreceptor assay (18, 20), our mPL preparation was $\approx$14 times more potent than mPRL in this assay. In addition, our mPL preparation stimulated the pigeon crop sac. Thus, given our present findings and those from other studies (5, 25, 26), we speculate that mPL may well be the lactogenic hormone of pregnancy in the mouse.

As observed in our studies on mPL, ovine (17, 19, 20), bovine (23), and human (27) PLs have also been shown not to crossreact with antisera raised against their own pituitary prolactin. On the other hand, bovine (23) and human (27) PLs were found to crossreact with antisera to their own pituitary growth hormone. In contrast, mPL and oPL (17, 20) did not crossreact with their corresponding antigrowth hormones.

The availability of purified mPL will now permit us to examine in detail the role of this hormone in pregnancy in a homologous system, the mechanisms involved in regulating its secretion, and additional biochemical properties of this molecule. We have generated a high-titer antiserum to mPL (50% binding of ^{125}I-labeled mPL at a dilution of 1:80,000) that can be used in future studies.

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