Isolation and Structural Characterization of Ovine Placental Lactogen†

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ABSTRACT: Ovine placental lactogen (oPL), a polypeptide hormone with biological properties similar to ovine prolactin (oPRL) and ovine growth hormone (oGH), has been purified from term placental cotyledons in an overall yield of 28% by a method which includes homogenization in absolute ethanol, pH and ammonium sulfate precipitation, gel chromatography, and ion-exchange chromatography on diethylaminoethyl- and carboxymethylcellulose. On electrophoresis in 7.5% polyacrylamide gels at pH 9.0, the hormone migrates as a single band (R_f 0.39) near the position of oGH. Electrophoresis in gels containing sodium dodecyl sulfate shows a single band with a mobility corresponding to a molecular weight of ~22 500. Aggregation of oPL was observed in buffers of low ionic strength (0.01 M Tris-HCl, pH 9.0) and at high concentration of the hormone (2.0 mg/mL). The isoelectric point of the monomer, determined by isoelectric focusing, is 6.8 ± 0.1; that of the aggregate is 7.7 ± 0.1. The amino acid composition of oPL, consisting of 193 residues, has general similarity to oGH and oPRL except for a low content of leucine and phenylalanine. Like oPRL, but in contrast to oGH, oPL contains 6 half-cystinyl and 2 tryptophanyl residues while its COOH-terminal sequence is the same as that of oGH: Cys-Ala-Phe-OH. Automated Edman degradation of performic acid oxidized oPL indicates amino-terminal heterogeneity, with alanine and valine in approximately equal amounts. The structural similarities to oPRL as well as oGH suggest that the evolutionary history of the subprimate placental lactogens differs from that of the primates.

In 1962, Josimovich and MacLaren isolated human placental lactogen (hPL;[1] human chorionic somatomammotropin, hCS) from term placentas and retroplacental blood and found that the hormone had activity comparable to that of prolactin in the pigeon crop assay. In addition, they showed that hPL reacted with antisera to human growth hormone (hGH) and stimulated epiphyseal growth in hypophysectomized rats with a potency about 1% that of hGH. Since then, structural studies have shown that hPL is a single chain polypeptide of 191 amino acids closely resembling hGH: 167 (87%) of the residue positions in the two proteins are occupied by identical amino acids; 18 of the remaining 24 residues are "highly acceptable replacements" (Bewley and Li, 1974). Wallis and Davies (1976) have pointed out that, with such similar amino acid sequences, hPL resembles hGH to a greater extent than does any other mammalian growth hormone of known sequence. This similarity of sequence has led to the proposal that hPL arose by a relatively recent duplication of the growth hormone gene (Dayhoff et al., 1975). Studies of the placental lactogen from the rhesus monkey (mPL) (Shome and Friesen, 1971; Vinik et al., 1973), though not as extensive as those on hPL, indicate that mPL also resembles the primate growth hormones.

Subprimate placental lactogens have not been as thoroughly studied, although evidence for their existence in some species was obtained by Ehrhardt as early as 1936. Later studies by Gusdon et al., (1970), Forsyth (1974), and others have produced evidence for the existence of a placental lactogen in a wide variety of subprimate species including the cow, goat, pig, sheep, deer, rat, mouse, hamster, vole, guinea pig, chinchilla, dog, rabbit, and horse. Nevertheless, purification and characterization of subprimate placental lactogens have been achieved only recently, with such hormones now purified from the rat (Robertson and Friesen, 1975), cow (Bolander and Fellows, 1976a), rabbit (Bolander and Fellows, 1976b), and sheep (Fellows et al., 1974; Martal and Djiane, 1975; Chan et al., 1976).

We have undertaken the purification of the placental lactogen from sheep (oPL) in order to investigate its structural relationship to growth hormone and prolactin (Hurley et al., 1975), to elucidate hormone-receptor interactions (Bolander et al., 1976), and to assess its biological and immunological properties (Handwerger et al., 1974), with development of the sheep as an animal model of placental lactogen physiology (Handwerger et al., 1975). Preliminary reports from this laboratory (Hurley et al., 1975; Fellows et al., 1976) have outlined purification methods for oPL which yield hormone of high purity but with relatively low recovery of total activity. In the method for isolation of oPL reported by Chan et al. (1976), the product was characterized primarily with respect to biological activity. This and the accompanying report describe a method of hormone purification which provides homogeneous oPL in greater yield as well as characterization of physical and chemical properties of oPL bearing on our understanding of the evolutionary origin of the subprimate placental lactogens.

Materials and Methods

Ovine growth hormone (NIH-GH-S9) and ovine prolactin (NIH-P-S-11), provided by the Hormone Distribution Pro-

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[1] Abbreviations used: hPL, human placental lactogen; hGH, human growth hormone; mPL, monkey placental lactogen; oPL, ovine placental lactogen; oPRL, ovine prolactin; OGH, ovine growth hormone; DEAE, diethylaminoethyl; CM, carboxymethyl; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; Pth, phenylthiohydantoin; SAE, *S*-aminoethyl.

gram, NIAMDD, were subjected to additional gel filtration (Fellows and Rogol, 1969). Sephadex G-150 (fine) was purchased from Pharmacia; microgranular, preswollen diethylaminoethyl (DEAE)-cellulose (DE52) and carboxymethylcellulose (CM52) were obtained from Whatman. Phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$) and Coomassie Brilliant Blue R were from Sigma. Sodium dodecyl sulfate ($NaDodSO_4$), ethylenimine, β-mercaptoethanol, *N*-ethylmorpholine, and phenylthiohydantoin (Pth)-amino acid standards ("sequanal grade") were from Pierce Chemical Co. Diisopropyl fluorophosphate-treated carboxypeptidase A (lot 35E634; specific activity 64 U/mg) and carboxypeptidase B (lot 34M668; specific activity 660 U/mg) were obtained from Worthington Biochemicals.

Acrylamide and *N,N*-methylenebisacrylamide (practical grade), purchased from Eastman Kodak Co., were recrystallized from hot chloroform (70 g/L) and hot acetone (10 g/L), respectively. Thin-layer chromatography plates were Eastman no. 6060, silica gel with fluorescent indicator. Ammonium sulfate ("enzyme grade") was purchased from Schwarz/Mann. Ultrapure hydrochloric acid (Ultrex, J. T. Baker Co.) was used for peptide hydrolyses. Reagents and solvents for amino acid and sequence analysis were from Beckman. Carrier-free $Na^{125}I$ was obtained from Amersham/Searle.

Ovine placental tissue was surgically removed from anesthetized ewes in the last 2 weeks of pregnancy and placed on ice. Cotyledons were dissected free, rinsed with cold, deionized water, and stored at −20 °C.

Radioreceptor Assays. The purification of oPL was monitored by a modification of the radioreceptor assay for lactogenic hormones developed by Shiu et al. (1973), using mammary tissues obtained from 30-day pregnant rabbits, ^{125}I-labeled ovine prolactin (oPRL) as tracer, and unlabeled oPRL as standard (Handwerger et al., 1975). The tracer was iodinated as described by Bolander and Fellows (1975).

Hormone Purification. All steps of the purification procedure were performed at 4 °C. Protein determinations (Lowry et al., 1951) and assays of radioreceptor activity were carried out on aliquots of each fraction.

Ovine placental cotyledons were broken into small pieces without thawing, rinsed with cold, deionized water, and weighed. They were then homogenized in 4–5 times their weight (V/W) of cold, absolute ethanol with a Waring blender and centrifuged at 850*g* for 45 min. The precipitate was air dried on a Buchner funnel after washing with 2–3 volumes of cold, absolute ethanol.

The dried cake was suspended in 10 times its weight (v/w) of 0.1 M NH_4HCO_3 containing 1.0 mM $PhCH_2SO_2F$. The pH was adjusted to 9.5 with concentrated NH_4OH and the suspension was stirred at 4 °C for 18 to 24 h. Insoluble material was removed by centrifugation. This and all subsequent centrifugation was at 8500*g* for 1 h. The pH of the crude extract was adjusted to 6.5 by the gradual addition of 6 N HCl and precipitate was removed by centrifugation. In this and subsequent steps, precipitation was judged to be complete when light scattering at 320 nm reached a plateau, in 30 to 40 min.

The pH 6.5 supernatant was fractionated by slow addition of solid ammonium sulfate. Material precipitating between 0.35 and 0.65 ammonium sulfate saturation was collected by centrifugation, dissolved in 0.1 M ammonium bicarbonate, pH 9.5, at approximately 60 mg/mL and applied to a column of Sephadex G-150 equilibrated and eluted with 0.1 M ammonium bicarbonate, pH 8.3.

Active material eluted from the Sephadex column was pooled and dialyzed against 0.01 M Tris-HCl, pH 9.0, until the retentate reached pH 9.0. This material was then pumped onto a column of DEAE-cellulose in 0.01 M Tris-HCl, pH 9.0. After initial elution with 5 column volumes of the equilibrating buffer, a linear gradient was begun with 0.01 Tris-HCl, pH 9.0, containing 0.05 M NaCl as the limit buffer.

Tubes containing activity were pooled and dialyzed against 0.01 M ammonium acetate, pH 5.5. The material was then pumped onto a column of carboxymethyl-cellulose in 0.01 M ammonium acetate, pH 5.5. After initial elution with equilibrating buffer, stepwise elution was begun with 0.01 M ammonium acetate containing 0.2 M NaCl. Tubes containing active material were pooled, dialyzed exhaustively against water, and lyophilized. This final purification product was used for all further studies.

Electrophoresis. Disc electrophoresis in 7.5% polyacrylamide separating gels, pH 9.0, was performed by the method of Ornstein (1964) and Davis (1964). Before $NaDodSO_4$ electrophoresis (Weber and Osborn, 1969), samples (50–125 μg) were incubated in 50–100 μL of a solution containing 0.05 M sodium phosphate, pH 7.2, 0.2% $NaDodSO_4$ with or without 2% β-mercaptoethanol, at 100 °C for 20 min. Electrophoresis was carried out at 2–3 mA per tube at room temperature. Gels were stained with Coomassie Brilliant Blue R, 0.25%, in water:acetic acid:methanol (5:1:5) for 15 min at 90 °C and destained in 7.5% acetic acid at 55 °C.

Isoelectric Point. Determinations were made at 10 °C using an LKB 2117 Multiphor apparatus and PAG plates containing broad (pH 3.5–9.5) or narrow (pH 5.0–8.5) range ampholytes. Focusing was begun at 200 V and continued until the voltage stabilized between 1100 and 1200 V, about 2 h. After the run, sections from the entire length of the gel were extracted overnight in 1.5 mL of boiled, deionized water. The pH of the extracts was measured at 10 °C. Proteins of known isoelectric point were run with samples to confirm the pH gradient. The plates were stained with Coomassie Brilliant Blue R, 0.25%, in water:methanol:trichloroacetic acid:sulfosalicylic acid (21:10:3.3:1) for 20 min at 60 °C and destained in water:ethanol:acetic acid (8:3:1) at room temperature.

Amino Acid Analysis. After hydrolysis of oPL for 24, 48, and 72 h in 6 N HCl at 110 °C under reduced pressure, the amino acid composition was determined on a Beckman 121 M microcolumn amino acid analyzer. The half-cystine content was determined as cysteic acid following oxidation with performic acid (Hirs, 1967); tryptophan was determined by the method of Edelhoch (1967).

Molecular Size. Molecular weight estimates were obtained by a comparison of the mobility of purified placental lactogen (1 mg/mL) with mobility of proteins of known molecular weight in polyacrylamide gels containing $NaDodSO_4$ (Weber and Osborn, 1969) and by a similar comparison of K_{av} obtained on elution from a column, 0.9 × 90 cm, of Sephadex G-150 equilibrated and eluted with 0.1 M ammonium bicarbonate, pH 8.5.

Amino Terminus. Degradation of the amino terminus of performic acid oxidized oPL was carried out with an updated Beckman Model 890B sequencer utilizing a 0.1 M Quadrol program and a combined benzene–ethyl acetate wash. Following manual conversion of the thiazolinone amino acids to phenylthiohydantoin (Pth) derivatives with 1 N HCl at 30 °C for 10 min, all Pth amino acids except histidine, arginine, and cysteic acid were identified and determined quantitatively on SP-400 columns with a Beckman GC-45 gas chromatograph. Identification based on retention time was confirmed by coelution with authentic Pth-amino acid standards. Further confirmation was obtained by thin-layer chromatography in two solvent systems: heptane–propionic acid–ethylene dichloride, 58:17:25 (Jeppson and Sjoquist, 1967), and xy-

TABLE I: Summary of the Purification of oPL.

Fraction	Total Protein (mg)[a]	Total Act. (mg)[b]	Recovery (%) Protein	Recovery (%) Act.	Spec Act.
Crude extract[c]	8614	13.9	100		0.002
pH 6.5/35% ammonium sulfate precipitate	1300	9.9	15		
35–65% ammonium sulfate precipitate	3690	76.9	43	100	0.021
65% ammonium sulfate supernatant	1120	11.2	13		
Chromatography on Sephadex G-150					
Pool 1	1972	6.9	53	9	
Pool 2	1124	34.2	30	44	0.030
Pool 3	403	0.2	11	1	
Chromatography on DEAE-cellulose					
Pool 2A	227	0.7	20	2	
Pool 2B	202	25.2	18	74	0.125
Pool 2C	124	0.5	11	1	
Pool 2D	565	6.1	50	18	
Chromatography on CM-cellulose					
Pool 2B1	66	0.4	33	1.5	
Pool 2B2	82	0.3	40	1	
Pool 2B3	21.4[d]	21.0	11	83	0.98

[a] Determined by the method of Lowry et al. (1951). [b] Determined in the radioreceptor assay for lactogens using oPRL as standard and tracer. [c] From a preparation begun with 500 g of frozen placental cotyledons. [d] Value based on amino acid analysis.

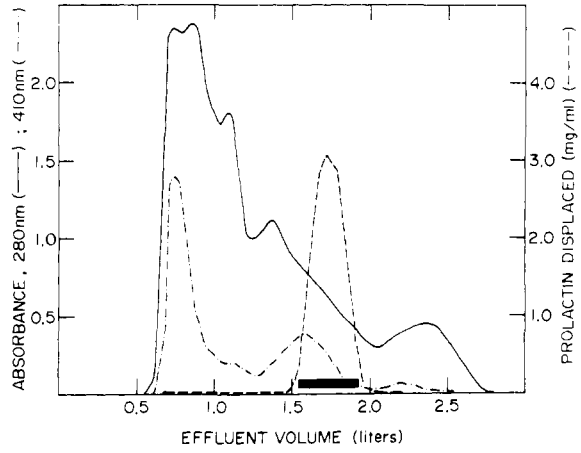

FIGURE 1: Chromatography of ovine placental lactogen on Sephadex G-150. After fractionation of the crude placental extract with ammonium sulfate, material precipitating between 0.35 and 0.65 saturation, 3690 mg, was applied in 50 mL of 0.1 M NH_4HCO_3 to a column, 5 × 125 cm, of Sephadex G-150 equilibrated and eluted at 4 °C with 0.1 M NH_4HCO_3 at 50 mL/h. Material indicated by the solid bar was pooled for further purification on DEAE-cellulose (Figure 2).

lene–methanol, 80:10 (Inagami and Murakami, 1972), as well as by the unique colors developed with ninhydrin, 0.5% in 1-butanol (Inagami and Murakami, 1972). The Pth derivatives of histidine, arginine, and cysteic acid, which remain in the aqueous phase after conversion, were identified by thin-layer chromatography in xylene–95% ethanol–acetic acid, 50:50:5 (Inagami and Murakami, 1972). In addition, Pth-histidine and Pth-arginine were identified by the Pauly and phenanthrenequinone reactions (Yamada and Itano, 1966), respectively.

Carboxyl Terminus. Residues at the COOH terminus of oPL were determined by digestion of *S*-aminoethyl oPL (SAE-oPL) with carboxypeptidases A and B at 37 °C (Ambler, 1967). SAE-oPL was prepared by dissolving unmodified protein (20 mg/mL) in 1.0 M Tris-HCl, pH 8.6, containing 2 mM EDTA and 8 M urea. β-Mercaptoethanol (30-fold molar excess over thiol groups) was added and the solution was incubated in a N_2 atmosphere for 4 h at room temperature. Ethylenimine equivalent to total thiol groups was added three times at 10-min intervals under N_2 (Cole, 1967). At 30 min, the reaction mixture was made 50% in acetic acid and applied directly to a column of Sephadex G-50 in 50% acetic acid.

SAE-oPL (38 nmol) was dissolved in 1.6 mL of 0.2 M *N*-ethylmorpholine acetate, pH 8.5, containing 2 mM $NaDodSO_4$ and incubated at 37 °C for 10 min. Carboxypeptidase A was added to an enzyme:substrate ratio of 1:50 (w/w). Aliquots of 4.7 nmol of SAE-oPL were removed at 0, 7.5, 15, and 30 min, and carboxypeptidase B was added to a final enzyme: substrate ratio of 1:15 (w/w). Additional 4.7-nmol aliquots of the digestion mixture were removed at 45, 60, 90, and 120 min. Each aliquot was made 5% in trichloroacetic acid, cooled at 0 °C for 2 h, and centrifuged at 750*g* for 5 min. Precipitates were washed once with 5% trichloroacetic acid. The combined supernatants of each aliquot were dried under a nitrogen stream, dissolved in 0.2 M sodium acetate (pH 3.2), and divided into two equal portions. One was applied to a Beckman 121M microcolumn amino acid analyzer; and the other was applied to a column, 0.28 × 19 cm, of Beckman AA-20 spherical resin equilibrated and eluted with 0.35 M sodium citrate, pH 5.26, in order to resolve SAE-cysteine from lysine. Results were corrected by blanks handled identically except that they contained no SAE-oPL.

Results

Hormone Purification. A summary of the purification of oPL is shown in Table I. Homogenization in cold, absolute ethanol was found to provide the best recovery and the best reproducibility in subsequent purification steps. Homogenization in aqueous buffer, on the other hand, was associated with less effective extraction, reduced solubility of the ammonium sulfate precipitates and poorer resolution during gel chromatography. When material insoluble in ethanol was thoroughly dried at room temperature under reduced pressure and extracted once with 0.1 M NH_4HCO_3, pH 9.5, the "crude extract" contained 70–80% of the total active material obtainable by multiple extractions.

oPL is eluted from Sephadex G-150 as a single symmetrical peak of activity in a position (K_{av} = 0.55) similar to that of oGH and oPR (Figure 1). Material absorbing at 410 nm provides a convenient marker in the absence of a distinctive

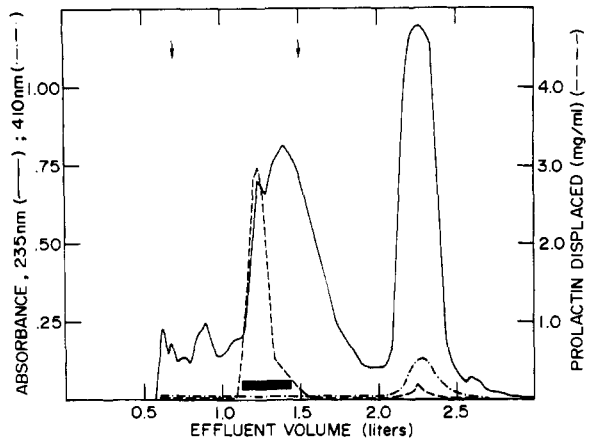

FIGURE 2: Chromatography of oPL on DEAE-cellulose. Material from G-150 chromatography was dialyzed against 0.01 M Tris-HCl, pH 9.0, and applied to a column, 2.5 × 35 cm, of DEAE-cellulose equilibrated in 0.01 M Tris-HCl, pH 9.0, at 4 °C. Elution was begun with the equilibrating buffer. At the first arrow, a linear gradient of 800 mL was begun using 0.01 M Tris-HCl, pH 9.0, containing 0.05 M sodium chloride as the limit buffer. At the end of the gradient elution was continued with the limit buffer and then changed, stepwise, at the second arrow to 0.01 M Tris-HCl, pH 9.0, with 1.0 M NaCl. The solid bar indicates material pooled for further purification on CM-cellulose (Figure 3).

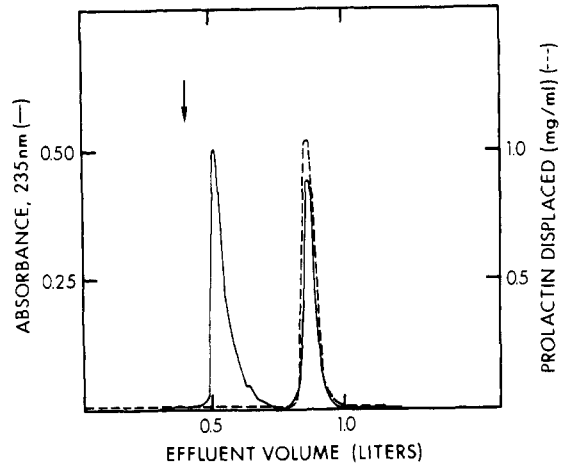

FIGURE 3: Chromatography of oPL on CM-cellulose. After dialysis against 0.01 M ammonium acetate, pH 5.5, the active material from DEAE chromatography was applied at 4 °C to a column, 2.5 × 20 cm, of CM-cellulose equilibrated and eluted with 0.01 M ammonium acetate, pH 5.5. At the arrow, stepwise elution was begun with 0.01 M ammonium acetate, pH 5.5, containing 0.2 M NaCl. The single, symmetrical peak of activity was pooled.

peak at 280 nm. When the active material, pooled as indicated in Figure 1, was dialyzed against 0.01 M Tris-HCl, pH 9.0, and pumped directly onto a column of DEAE-cellulose, it was eluted as a single peak by a gradient to 0.05 M NaCl (Figure 2). The minor peak, consistently seen following 1.0 M NaCl elution, was not further characterized. When active material, pooled as indicated in Figure 2, was applied directly to a CM-cellulose column after dialysis against 0.01 M ammonium acetate, pH 5.5, a stepwise change to buffer containing 0.2 M sodium chloride resulted immediately in elution of a large amount of protein followed by the single symmetrical peak of oPL activity (Figure 3). This material was used for characterization studies.

While aggregation of oPL is not evident in the 0.1 M ammonium bicarbonate buffer routinely used for gel chromatography, it is apparent in buffer of lower ionic strength (Figure 4). When the 0.35–0.65 ammonium sulfate precipitate is eluted from a G-150 column with 0.01 M Tris-HCl, pH 9.0, peaks with $K_{av} = 0.55$ and $K_{av} = 0.22$ are both detected by radioreceptor assay. When the early eluting material was pooled as indicated and rechromatographed on a column of G-150 in 0.1 M ammonium bicarbonate, pH 9.0 (Figure 4, lower), its elution position was the same as the retarded peak (Figure 4, upper) and the active peak of Figure 1 ($K_{av} = 0.55$).

Electrophoresis. In disc gels at pH 9.0 and 7.5% acrylamide concentration (Figure 5, gel C), oPLmigrates as a single band with a R_f of 0.39, similar to one band of oGH (gel B: R_f 0.48, 0.39, 0.27) but slower than the bands of oPRL (gel A: R_f 0.84, 0.77, 0.68). In the presence of $NaDodSO_4$ (gel D), only one band is observed. In disc gels without $NaDodSO_4$, it was found that bands with R_f 0.31 and 0.25 were generated as a function of initial sample concentration: gel C is loaded with 75 μg of oPL dissolved at 0.5 mg/mL in Tris–glycine buffer, while gel E is loaded with 75 μg of the same oPL preparation dissolved at 5.0 mg/mL in the same buffer.

Molecular Size. When the mobility of oPL in $NaDodSO_4$ gels is compared with that of proteins of known molecular weights, a molecular weight of ~22 500 is estimated. A somewhat higher estimation, 27 300, is obtained after chromatography on Sephadex G-150 with 0.1 M ammonium bicarbonate. In both systems, oPL moves at the positions of oGH and oPRL (molecular weights ~22 000).

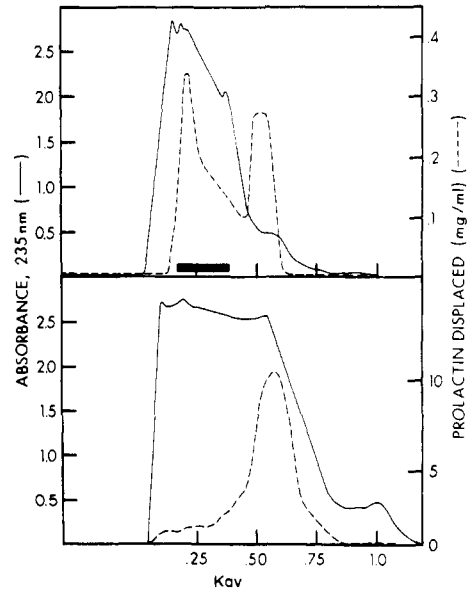

FIGURE 4: The effect of ionic strength on elution volume of oPL during chromatography on Sephadex G-150. The upper panel shows the elution of material contained in the 35–65% ammonium sulfate precipitate from a column, 5 × 125 cm, of Sephadex G-150 equilibrated and eluted at 4 °C with 0.01 M Tris-HCl, pH 9.0. Material eluting early, as indicated by the solid bar, was pooled and reapplied to a column, 2.5 × 90 cm, of Sephadex G-150 equilibrated and eluted in 0.1 M $(NH_4)HCO_3$, pH 9.0. Elution of the reapplied material is shown in the lower panel.

Isoelectric Point. Gel electrofocusing at a sample concentration of 0.5 mg/mL in the pH ranges of 3.5 to 9.5 and 5.5 to 8.0 indicated an isoelectric point of 6.8 ± 0.1. Since oPL is known to aggregate in conditions of low ionic strength and high protein concentration (Figures 4 and 5), electrofocusing was repeated with oPL at 2 mg/mL in water. The isoelectric point of the aggregate is pH 7.7 ± 0.1.

Amino Acid Composition. The amino acid composition of oPL is compared with those of oGH and oPRL in Table II, where the general similarity is apparent. Although oPL is not identical with oGH in content of any amino acid, it is closer

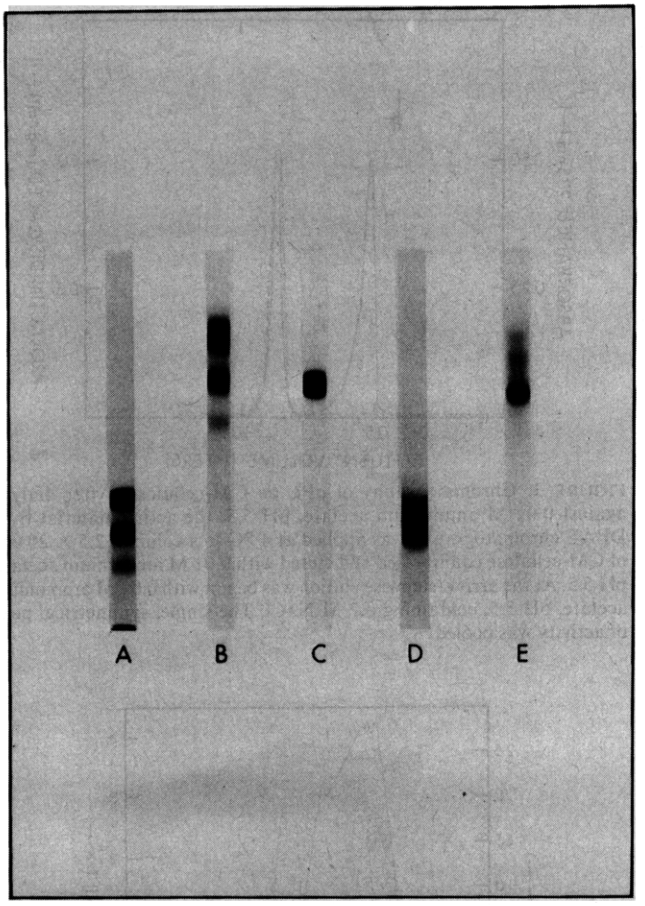

FIGURE 5: Electrophoresis of oPL in polyacrylamide disc and $NaDodSO_4$ gels. Electrophoresis was carried out in 7.5% separating gels at room temperature at 2–3 mA per gel. Direction of migration (the anode) was downward. A, B, C, and E are all disc gels, pH 9.0, without $NaDodSO_4$ and are loaded with 75 μg each of oPRL (gel A), oGH (gel B), oPL at 0.5 μg/mL initial concentration (gel C), and oPL at 5.0 mg/mL initial concentration (gel E). Gel D is of 7.5% polyacrylamide containing $NaDodSO_4$ and was loaded with 125 μg of oPL.

TABLE II: The Amino Acid Compositions of oPL, oGH, and oPRL.[a]

Amino acid	oPL	oGH[b]	oPRL[c]
Lys	14.1 (14)	13	9
His	4.0 (4)	3	8
Arg	10.0 (10)	13	11
Asp	19.1 (19)	16	22
Thr	10.3 (10)[d]	12	9
Ser	14.8 (15)[d]	12	15
Glu	23.6 (24)	25	23
Pro	9.9 (10)	8	11
Gly	14.6 (15)	10	11
Ala	12.8 (13)	14	9
½-cystine	5.8 (6)[e]	4	6
Val	12.2 (12)	7	10
Met	4.9 (5)	4	7
Ile	10.2 (10)	7	11
Leu	12.9 (13)	22	22
Tyr	3.6 (4)	6	7
Phe	7.0 (7)	13	6
Trp	2.1 (2)[f]	1	2
Total	193	191	199
Calculated mol. wt	21418	21806	22720

[a] Values for amino acids are expressed as residues per molecule with nearest integral numbers in parentheses. [b] From Wilhelmi (1974). [c] From Li (1972). [d] Extrapolated to zero time of hydrolysis. [e] Determined as cysteic acid after oxidation with performic acid. [f] Determined by the spectrophotometric method of Edelhoch (1967).

TABLE III: Amino-Terminal Sequence Analysis of Performic Acid-Oxidized oPL.[a]

Pth-amino acid	Cycle no. 1	2	3	4	Total
Ala	15	0	0		15
Val	17	4	0		21
Phe	0	18	9		27
Cysteic acid	0	+	+++	+	

[a] Amounts of each residue are expressed in nanomoles.

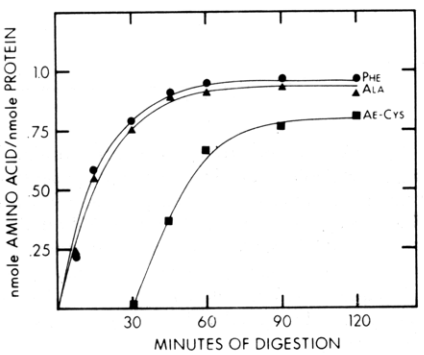

FIGURE 6: Digestion of *S*-aminoethyl oPL with carboxypeptidases A and B. *S*-Aminoethyl oPL (38 nm) was dissolved in 0.2 M *N*-ethylmorpholine acetate (pH 8.5) containing 2 mM $NaDodSO_4$ and digested with carboxypeptidase A at 37 °C. Aliquots of 4.7-nm *S*-aminoethyl oPL each were removed from the digestion mixture after 7.5, 15, and 30 min of reaction. After removal of the aliquot at 30 min, carboxypeptidase B was added and digestion continued for 90 more min with removal of aliquots at the times shown. Protein was separated from free amino acids by precipitation of each aliquot with trichloroacetic acid. The supernatants were dried under nitrogen and amino acids determined on a Beckman 121M Microcolumn amino acid analyzer.

to oGH than to oPRL in numbers of lysine, histidine, aspartic acid, alanine, and tyrosine. oPL is closer to oPRL in content of arginine, threonine, proline, valine, isoleucine, and phenylalanine and is identical with oPRL in its content of serine, half-cystine, and tryptophan. The estimated total number of residues in oPL (193) is intermediate between oGH (191) and oPRL (199). The minimum molecular weight of oPL, calculated from its amino acid composition, is 21 418.

Carboxyl Terminus. Figure 6 illustrates the results of digestion of SAE-oPL with carboxypeptidases A and B. Phenylalanine and alanine are released at similar rates with SAE-cysteine released more slowly than either. No other amino acids were detected during the incubation period.

Amino Terminus. Degradation of performic acid oxidized oPL (35 nmol) by the automated Edman procedure revealed heterogeneity at the amino terminus. The Pth-amino acids detected in the first four Edman cycles are summarized in Table III. No amino acids were detected beyond the fourth cycle.

In the first cycle, Pth-alanine (15 nmol) and Pth-valine (17 nmol) are present in nearly equal amounts. At cycle two, Pth-phenylalanine (18 nmol) is found in amounts nearly the same as those observed for each of the amino acids in the first cycle. Also in the second cycle is a small amount of Pth-valine and the first appearance of Pth-cysteic acid detected by thin-layer chromatography. The third cycle shows only Pth-phenylalanine (half as much as was found in the preceding cycle) and Pth-cysteic acid in greater amount than was found in cycle

2. The fourth cycle reveals only a reduced amount of Pth-cysteic acid.

Discussion

An important factor in maintaining biological activity of oPL is the handling and storage of placental tissue. These studies have used oPL obtained from placentas delivered surgically from live sheep near term, with cotyledons dissected, rinsed and frozen immediately. We have found (unpublished observations) that, when oPL is obtained from cotyledons collected within 30 min of normal delivery, behavior in the purification procedure is unaltered but activity in radioreceptor and radioimmunoassays is only 1–2% that of oPL obtained from surgically delivered placentas.

Earlier purifications (Hurley et al., 1975; Fellows et al., 1976), making use of selective pooling of the DEAE column fractions, resulted in relatively high specific activity (0.33–0.89) but poor recovery of total activity (12–32%) at that step. The purification procedure detailed here is preparative in scale and provides better recoveries of homogeneous oPL which is equipotent with prolactin in the lactogenic receptor assay (Table I).

Two significant changes in total radioreceptor activity occur in the course of purification (Table I). The 0.35–0.65 ammonium sulfate precipitate has a total activity more than five times that of the crude extract, suggesting that material in the crude extract interfering with the detection of oPL by radioreceptor assay is removed or rendered noninterfering by precipitation. The second major change is the loss of more than 50% of total activity during chromatography on Sephadex G-150. Degradation by proteolytic enzymes is possible since the material applied to the column contains more than 40% of the protein in the crude extract. Partial inactivation seems unlikely since the final product of purification is equipotent with oPRL in the radioreceptor assay for lactogens, consistent with our preliminary report (Handwerger et al., 1974) and the study of Chan et al. (1976). In spite of this loss, oPL is obtained in overall yield of 28%.

Aggregation, especially in alkaline buffers, is characteristic of growth hormone-like proteins. Studies of bGH (Dellacha et al., 1968), hPL (Belleville et al., 1975), and bPL (Bolander and Fellows, 1976a) have all shown aggregation of the hormones. oPL aggregates reversibly in low ionic strength buffers (Figure 4) and at high protein concentration (Figure 5C and E). Electrofocusing experiments in which oPL was applied at high concentrations in water show that the p*I* of aggregated oPL is 7.7 ± 0.1, in contrast to the p*I* of 6.8 ± 0.1 for the monomeric form. Since oPL aggregates in the buffer in which it is applied to the DEAE column, the finding that the aggregate has a higher isoelectric point than the monomer can explain the two peaks of activity eluted from DEAE-cellulose (Figure 2). Since 0.05 M sodium chloride displaces the principal peak of activity from the DEAE column, its net negative charge at pH 9.0 should be less than that of the minor peak eluted with 1.0 M NaCl. This suggests that the former is in the aggregate form (p *I* 7.7) and that the smaller amount of activity eluted by high salt concentrations represents monomeric oPL (p*I* = 6.8). In all other purification steps, oPL behaves as a single species.

Comparison of the mobilities of oPL, oGH, and oPRL in polyacrylamide gels containing $NaDodSO_4$ indicates that all three hormones are of similar size, corresponding to molecular weights of 21 000–23 000. Stokes radii estimated by chromatography on Sephadex G-150 under nondenaturing conditions are also similar. Thus, purified oPL, like oGH and oPRL, appears to exist as a single polypeptide chain with a molecular weight of about 22 000. The molecular weight calculated from the amino acid composition (21 418, Table II) is in good agreement.

Though their apparent molecular weights in solution are similar, the amino acid compositions of the three hormones show important differences (Table II). Notable are the much lower amount of leucine (13 for oPL; 22 each for oGH and oPRL) and phenylalanine (7 for oPL; 13 for oGH). The large differences in hydrophobic amino acids may explain the greater solubility of oPL relative to oGH in aqueous buffers.

The amino acid composition also indicates that oPL is closely related to both oPRL and oGH. Unlike hPL, which is nearly identical with hGH (Li, 1972), oPL more closely resembles oPRL (and differs from oGH) in several important respects, including identical numbers of half-cystine and tryptophan residues. Of all the amino acids, half-cystine and tryptophan are considered to be the least subject to accepted mutation in the course of protein evolution (Dayhoff and Barker, 1972) and thus are of particular relevance in evaluating evolutionary relationships among proteins.

When oPL is digested with carboxypeptidases A and B (Figure 6), phenylalanine and alanine are released at about the same rates. Though this is consistent with the specificity of the enzyme, it makes interpretation difficult. Sequence data from the carboxyl terminus are available for two other placental lactogens. That of hPL (Li, 1972) is Cys-Gly-Phe-OH, while that of bovine placental lactogen (bPL, Bolander and Fellows, 1976a,b) is Cys-Ala-Phe-OH. In both cases, the sequence is identical with that of the respective growth hormone. The carboxyl terminus of oGH, Cys-Ala-Phe-OH (Wilhelmi, 1974), is consistent with the data obtained for oPL and the same sequence probably occurs at the carboxyl terminus of oPL.

Amino-terminal heterogeneity has been observed with several growth hormone-like proteins including bGH (Fellows et al., 1972), hPL (Catt et al., 1967), and bPL (Bolander, 1976). The heterogeneity observed in the amino-terminal sequence analysis of performic acid oxidized oPL (Table III) is consistent with the presence of two peptide chains in approximately equal amounts, one beginning with valine, the other with alanine.

A cystine residue near the amino terminus is a characteristic structural feature of the prolactins not found in the mammalian growth hormones (Li, 1972; Wilhelmi, 1972). The presence of a half-cystine residue near the amino terminus of oPL thus offers additional evidence for a close evolutionary relationship between oPL and oPRL and suggests that the placental lactogens of subprimates may have evolved from a precursor in the prolactin line rather than from growth hormone, as proposed for the primate placental lactogens.

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References

Ambler, R. P. (1967), *Methods Enzymol. 11*, 155–166.

Belleville, F., Peltier, A., Paysant, P., and Nabet, P. (1975), *Eur. J. Biochem. 51*, 429–435.

Bewley, T. A., and Li, C. H. (1974), in Lactogenic Hormones, Fetal Nutrition and Lactation, Josimovich, J. B., Ed., New York, N.Y., Wiley, pp 19–32.

Bolander, F. F. (1976), Doctoral Dissertation, Duke Univer-

sity.
Bolander, F. F., and Fellows, R. E. (1975), *Biochemistry 14*, 2938–2943.
Bolander, F. F., and Fellows, R. E. (1976a), *J. Biol. Chem. 251*, 2703–2708.
Bolander, F. F., and Fellows, R. E. (1976b), *Biochem. J. 159*, 775.
Bolander, F. F., Hurley, T. W., Handwerger, S., and Fellows, R. E. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73*, 2932–2935.
Catt, K. J., Moffat, B., and Niall, H. (1967), *Science 157*, 321.
Chan, J. S. D., Robertson, H. A., and Friesen, H. G. (1976), *Endocrinology 98*, 65–76.
Cole, R. D. (1967), *Methods Enzymol. 11*, 315–317.
Davis, B. J. (1964), *Ann. N.Y. Acad. Sci. 121*, 404–427.
Dayhoff, M. O., and Barker, W. C. (1972), in Atlas of Protein Sequence and Structure, Dayhoff, M. O., and Barker, W. C., Ed., Silver Spring, Md., The National Biomedical Research Foundation, p 17.
Dayhoff, M. O., McLaughlin, P., Barker, W., and Hunt, L. (1975), *Naturwissenschaften 62*, 154–161.
Dellacha, J. M., Santome, J. A., and Paladini, A. C. (1968), *Ann. N. Y. Acad. Sci. 148*, 313–327.
Edelhoch, H. (1967), *Biochemistry 6*, 1948–1954.
Ehrhardt, K. (1936), *Muench. Med. Wochenschr. 83*, 1163–1164.
Fellows, R. E., Bolander, F. F., Hurley, T. W., and Handwerger, S. (1976), *Excerpta Med. Found. Int. Cong. Ser., No. 381*, 392–403.
Fellows, R. E., Hurley, T., Maurer, W., and Handwerger, S. (1974), *Endocrinology 94*, A110.
Fellows, R. E., and Rogol, A. D. (1969), *J. Biol. Chem. 244*, 1567–1575.
Fellows, R. E., Rogol, A. D., and Mudge, A. W. (1972), *Excerpta Med. Found. Int. Cong. Ser., No. 244*, 392–403.
Forsyth, I. A. (1974), in Lactogenic Hormones, Fetal Nutrition and Lactation, Josimovich, J. B., Ed., New York, N.Y., Wiley, pp 49–67.
Gusdon, J. P., Leake, N. H., Van Dyke, A. H., and Atkins, W. (1970), *Am. J. Obstet. Gynecol. 107*, 441–444.
Handwerger, S., Maurer, W., Barrett, J., Hurley, T. W., and Fellows, R. E. (1974), *Endocr. Res. Commun. 1*, 403–413.
Handwerger, S., Maurer, W., Crenshaw, M. C., Hurley, T. W., Barrett, J., and Fellows, R. E. (1975), *J. Pediatr. 87*, 1139–1143.
Hirs, C. H. W. (1967), *Methods Enzymol. 11*, 59–62.
Hurley, T. W., Maurer, W., Handwerger, S., and Fellows, R. E. (1975), in Peptides: Chemistry, Structure and Biology, Walter, R., and Meienhoffer, J., Ed., Ann Arbor, Mich., Ann Arbor Science Publishers, pp 583–588.
Inagami, T., and Murakami, K. (1972), *Anal. Biochem. 47*, 501–504.
Jeppson, J.-O., and Sjoquist, J. (1967), *Anal. Biochem. 18*, 264–269.
Josimovich, J. B., and MacLaren, J. A. (1962), *Endocrinology 71*, 209–220.
Li, C. H. (1972), in Lactogenic Hormones, Wolstenholme, G. E. W., and Knight, J., Ed., Edinburgh, Churchill-Livingstone, pp 7–26.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Martal, J., and Djiane, J. (1975), *Biochem. Biophys. Res. Commun. 65*, 770–778.
Ornstein, L. (1964), *Ann. N.Y. Acad. Sci. 121*, 321–349.
Robertson, M. C., and Friesen, H. G. (1975), *Endocrinology 97*, 621–629.
Shiu, R. P. C., Kelly, P. A., and Friesen, H. G. (1973), *Science 180*, 968–971.
Shome, B., and Friesen, H. G. (1971), *Endocrinology 89*, 631–641.
Vinik, A. I., Kaplan, S. L., and Grumbach, M. M. (1973), *Endocrinology 88*, 1349–1352.
Wallis, M., and Davis, R. V. (1976), *Excerpta Med. Found. Int. Cong. Ser., No. 381*, 1–13.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406–4412.
Wilhelmi, A. E. (1974), in Handbook of Physiology, Section 7, Vol. IV, Part 2, Greep, R. O., and Astwood, E. B., Ed., Washington, D.C., American Physiological Society, pp 59–78.
Yamada, S., and Itano, H. (1966), *Biochim. Biophys. Acta 130*, 538–540.