PURIFICATION AND SOME PROPERTIES OF OVINE PLACENTAL LACTOGEN

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(Received 12 September 1977)

SUMMARY

A method has been described for the purification of ovine placental lactogen (oPL) involving the use of freshly obtained sheep foetal cotyledons. Tissue was extracted with 0·1 m-ammonium bicarbonate and the supernatant fraction, adjusted to pH 7, was brought to 60% saturation with ammonium sulphate. The resulting precipitate was then subjected to a sequence of chromatographic steps using columns of Sephadex G-100 and carboxymethylcellulose. During each stage of the purification, the lactogenic activity was monitored with a pregnant rabbit mammary gland radioreceptor assay. The yield of oPL corresponded to 8 mg/kg wet foetal tissue and the oPL possessed lactogenic activity equivalent to 1 mg ovine prolactin/mg protein and GH-like activity equivalent to 0·8 mg human GH/mg protein. The biological activity of oPL was confirmed using a rabbit intraductal mammary gland assay in vivo.

After polyacrylamide gel electrophoresis at pH 8·9, oPL was resolved into one major band (isoelectric point 8·2–8·4) and four minor components, which were thought to be deamidation products of oPL. Microimmunoelectrophoresis and immunodiffusion studies confirmed that the preparation of oPL was free from serum protein contaminants.

INTRODUCTION

Astwood & Greep (1938) demonstrated that the rodent placenta elaborates a substance(s) essential for the maintenance of the corpus luteum. Other workers have studied the nature of protein(s) present in the rat placenta which cause lactogenesis in mammary gland tissue in vitro and in vivo (Leonard, 1945; Averill, Ray & Lyons, 1950; Matthies, 1968; Gusdon, Leake, van Dyke & Atkins, 1970). More recently Shiu, Kelly & Friesen (1973) developed a radioreceptor assay for proteins possessing prolactin-like activity, which used membranes isolated from rabbit mammary glands.

With the aid of the mammary gland radioreceptor assay, it has now been possible to purify rat placental lactogen (Robertson & Friesen, 1975), as well as the placental lactogens from a number of non-primate species, e.g. sheep (Handwerger, Maurer, Barrett, Hurley & Fellows, 1974; Hurley, Maurer, Handwerger & Fellows, 1975; Martal & Djiane, 1975; Chan, Robertson & Friesen, 1976; Fellows, Bolander, Hurley & Handwerger, 1976), cow (Bolander & Fellows, 1976a) and rabbit (Bolander & Fellows, 1976b).

In this paper, we describe a convenient method for the purification of ovine placental lactogen (oPL) and compare some of its biochemical and immunological properties with those reported by others. A short account of this work has been presented elsewhere (Reddy & Watkins, 1977).

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MATERIALS AND METHODS

Hormone preparations
Ovine prolactin (NIH-P-S7, 24·3 i.u./mg), ovine growth hormone (GH, NIH-GH-S7, 0·81 i.u./mg), bovine GH (NIH-GH-B17, 0·92 i.u./mg) and human GH (NIH-GH-HS1863, 1·6 i.u./mg) were kindly supplied by NIAMMD, National Institutes of Health, U.S.A. Samples of highly purified ovine and bovine GH were gifts from Dr C. H. Li, Hormone Research Laboratory, University of California, San Francisco, U.S.A. Human placental lactogenic (hPL) was a product of Nutritional Biochemicals Corporation, Ohio, U.S.A., Batch No. 2464.

Protein determinations
Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Iodination of hormones
Ovine prolactin and human GH were iodinated by the lactoperoxidase technique (Frantz & Turkington, 1972) and purified by passage through a column (1 cm × 50 cm) of Sephadex G-75 using 25 mM-Tris–HCl buffer containing 10 mM-MgCl₂ and 0·1% (w/v) bovine serum albumin as eluant at a flow rate of 10 ml/h. The radioactivity in each 1 ml fraction was measured in a Nuclear Chicago γ-spectrometer and those fractions showing high receptor-binding activity (see below) were diluted with 1 ml column buffer and stored at −20 °C. The specific activity of the labelled ovine prolactin and human GH ranged from 100 to 130 Ci/g (Berson, Yalow, Bauman, Rothschild & Newerly, 1956).

Radioreceptor assays
Lactogenic activity was assayed by a minor modification of the method of Shiu et al. (1973), using a subcellular fraction prepared from the mammary glands of 28–30 day pregnant New Zealand White rabbits (6–9 months old). Incubation was at room temperature (20 °C) for 16–18 h. Bound and free hormone was separated by centrifugation at 4 °C.

Human GH-like activity of protein fractions was determined by a radioreceptor assay (Tsushima & Friesen, 1973). Receptor protein (75–100 µg/tube) was prepared from the livers of the same pregnant rabbit and incubated with 125I-labelled human GH. The experimental procedure was as described for the measurement of lactogenic activity.

Radioreceptor assays were carried out on three dilutions of the protein samples, confirming the presence of parallelism.

Extraction of placental tissue
Unless otherwise stated, purification of oPL was carried out at 4 °C. Placental tissue was collected from 120 to 140 day pregnant ewes after slaughter by severance of the jugular vein. Within 30 min of death, the foetal cotyledons were separated from the maternal component, rinsed in ice-cold water to remove blood and stored at −20 °C.

Foetal cotyledons (1 kg) thawed to 4 °C were cut into approximately 2 cm cubes and homogenized in 3 litres 0·1 M-NH₄HCO₃ (pH 8·5) using an Ultraturrex T-45 Homogenizer (Janke & Kunkel Co., Germany) operated for 5–6 min. The homogenate was extracted for 21 h and then centrifuged at 1000 g for 15 min to remove tissue debris. Glacial acetic acid was added to reduce the pH of the supernatant fraction to 5·2, the resulting precipitate was removed by centrifugation at 1000 g for 15 min and the supernatant fraction was centrifuged again at 15 000 g for 10 min. The supernatant fluid obtained after the second centrifugation was adjusted to pH 7 with NH₄OH solution and then brought to 60% saturation by the slow addition of powdered ammonium sulphate. After 12 h, the precipitate, collected by centrifugation at 27 000 g for 15 min, was redissolved in 800 ml 0·1 M-NH₄HCO₃, pH 8·5, dialysed against the same buffer for 20 h (3 × 3 litres) using Visking 18/32 tubing and then freeze-dried.
Purification of ovine placental lactogen

Column chromatography
Further purification of oPL involved a series of column chromatographic steps. Protein obtained by ammonium sulphate precipitation of the placental extract was initially chromatographed on a column of Sephadex G-100 (Text-fig. 1) and the protein with lactogenic activity rechromatographed on a column of carboxymethylcellulose (Text-fig. 2). The final purification step involved the use of a second column of Sephadex G-100 (Text-fig. 3).

Polyacrylamide gel electrophoresis
Polyacrylamide gel electrophoresis was carried out in tubes of 7-5% acrylamide polymerized in Tris–HCl buffer, pH 8.9, according to the method of Davis (1964). The gels were stained with Coomassie Blue in 12% (w/v) trichloroacetic acid (Chrambach, Reisfeld, Wyckoff & Zaccari, 1967).

For determining the distribution of lactogenic activity in the polyacrylamide matrix, duplicate gels containing oPL were subjected to electrophoresis after which time one gel was stained for protein. The other gel was cut into 1 mm slices and each slice was extracted with 250 µl 25 mM-Tris–HCl buffer, pH 7.6, containing 10 mM-MgCl₂ and 0-1% (w/v) bovine serum albumin, for 21 h. The supernatant fraction was assayed for prolactin-like activity using the radioreceptor assay.

Determination of isoelectric point
Analytical isoelectric focusing was performed on polyacrylamide gels (0.8 cm × 20 cm) using a method based on that described by Wrigley (1971). The gels contained 2% ampholyte (pH range 3.5–10; Ampholine LKB-Produkter AB Sweden, Batch No. 16) in the presence of 6-25% acrylamide and a sample (3 mg) of oPL. After each gel was run at a maximum power of 1.5 W for 20 h, slices (1 cm) were cut and extracted as described above. The extract was assayed by the human GH receptor assay. A second gel, run simultaneously, was extracted with 1.5 ml distilled water for 18 h and the pH of the resulting extracts measured.

Molecular weight determination
The molecular weight of oPL was estimated by gel filtration (Andrews, 1969) using a column of Sephadex G-100 loaded with oPL (15 µg), ¹²⁵I-labelled ovine prolactin (1 × 10⁴ counts/min) and standard proteins (10 mg of each) and eluted with buffer at a flow rate of 12 ml/h. Fractions (3 ml) were counted for radioactivity, assayed for lactogenic activity and the positions of the protein peaks determined by measuring the ultraviolet absorbance of the effluent at 280 nm.

For each standard protein the partition coefficient \( K_{AV} \) was calculated from the elution volume, \( V_e \), the bed volume of the column, \( V_t \), and the void volume, \( V_0 \), by the expression

\[
K_{AV} = (V_e - V_0)/(V_t - V_0).
\]

From the plot of \( K_{AV} \) against the logarithm of the molecular weight of the protein samples, it was possible to estimate the molecular weight of oPL.

Preparation of anti-oPL serum
Anti-oPL serum was raised in male New Zealand White rabbits. On each occasion, subcutaneous injections of 250 µg oPL in 1 ml 0-1 M-NH₄HCO₃, pH 8.5, emulsified with 1 ml Freund’s complete adjuvant were given at multiple sites in the dorsal area of each rabbit at 2 week intervals for 8 weeks. Blood was collected via the ear vein 1 week after the final injection and the serum stored at -20 °C. All rabbits injected gave satisfactory antibodies.
Microimmunodiffusion and microimmunoelectrophoresis

Microimmunodiffusion was carried out on glass slides coated with 1 ml agar. For details of the reactants, see Pl. 2, fig. 2.

Microimmunoelectrophoresis was performed on glass plates (8 cm × 8 cm) coated with 11 ml 1-75% (w/v) agar in 45 mm-sodium barbitone buffer, pH 8-6. The wells (1 mm diameter) contained either normal sheep serum or oPL; electrophoresis was carried out for 60 min at 120 V using an apparatus supplied by Helena Laboratories, Texas, U.S.A. Immunodiffusion was then carried out for 24 h after placing either anti-sheep serum (Dakopatts A/S Denmark, Lot 043) or anti-oPL serum into troughs (1 mm × 50 mm) cut 5 mm from the wells.

Biological activity of oPL

The method used to establish the biological activity of oPL was the rabbit intraductal mammary gland assay performed in vivo (Lyons, 1942; Handwerger et al. 1974). Four days after the injection of the test sample, the mammary tissue immediately surrounding each test nipple was excised and fixed in a mixture of 10 ml formaldehyde (40%, w/v), 5 ml acetic acid and 100 ml ethanol (70%, v/v) for 48 h. After dehydration through a graded alcohol series and embedding in paraffin wax, 8 μm sections were cut and stained with haematoxylin and eosin.

RESULTS

Tissue extraction

From 1 kg sheep foetal cotyledons extracted with NH4HCO3, 50 g protein were obtained containing 116 mg lactogenic activity in terms of the ovine prolactin standard. After precipitation with 60% ammonium sulphate, 20% of the protein and 97% of the lactogenic activity were recovered (Table 1).

Table 1. Summary of the yields obtained during the various stages of purification of ovine placental lactogen (oPL) from 500 g wet placental tissue

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Protein (mg)</th>
<th>oPL (mg)*</th>
<th>Recovery (%)</th>
<th>Specific activity (mg prolactin activity/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium bicarbonate extraction</td>
<td>25 109†</td>
<td>58-6</td>
<td>100</td>
<td>0-002</td>
</tr>
<tr>
<td>Ammonium sulphate precipitation  (60%)</td>
<td>5000†</td>
<td>56-8</td>
<td>20</td>
<td>0-011</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>462-0‡</td>
<td>45</td>
<td>1-84</td>
<td>76-75</td>
</tr>
<tr>
<td>Carboxymethylcellulose</td>
<td>15-3‡</td>
<td>13-2</td>
<td>0-06</td>
<td>22-5</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>4‡</td>
<td>4</td>
<td>0-015</td>
<td>6-8</td>
</tr>
</tbody>
</table>

* Calculated from the radioreceptor assay using ovine prolactin as standard.
† Determined by the procedure of Lowry et al. (1951).
‡ Determined from dry weight.

Gel exclusion and ion-exchange chromatography

Text-figure 1 represents the elution pattern for protein and oPL when a sample of the ammonium-sulphate-precipitated material was passed through a column of Sephadex G-100. Although most fractions were associated with some lactogenic activity, 79-2% of the prolactin-like activity (based upon the ovine prolactin standard) was eluted as a single peak immediately after the emergence of the darkly coloured compound corresponding to a fraction number of 450. Protein in the former peak was pooled and after freeze-drying gave a light brown powder. We consider the presence of prolactin-like activity in
Purification of ovine placental lactogen

Text-fig. 1. Elution of 5 g protein, obtained from an extract of sheep foetal cotyledons by precipitation with 60% (w/v) ammonium sulphate, on a column (10 cm × 100 cm) of Sephadex G-100. The eluant was 0-1 m-NH₄HCO₃ at a flow rate of 70 ml/h. Fractions (11·6 ml) were assayed for lactogenic activity (hatched area). Ultraviolet absorption (E) at 280 nm was also measured (○); oPL, ovine placental lactogen.

higher-molecular-weight regions to be due to interference by proteinaceous material, giving rise to false high values. Similar effects were reported by Robertson & Friesen (1975).

Protein collected from the large column of Sephadex G-100 was fractionated by cation-exchange chromatography on carboxymethylcellulose; the elution pattern is shown in Text-fig. 2. Of the material applied to the column, almost 99% was either unabsorbed or eluted with 0·1 m-NaCl. Three peaks of lactogenic activity were eluted: a minor fraction (peak A) emerging at the end of the 0·1 m-NaCl step, peak B, eluted with 0·15 m-NaCl and a third peak (C) eluted with 0·2 m-NaCl. Although the quantities of protein recovered from peaks B and C were similar, the specific activity of the former (0·86 mg prolactin/mg protein) was greater than the latter (0·25 mg prolactin/mg protein).

Text-fig. 2. Ion-exchange chromatography on carboxymethyl cellulose of the lactogen (463 mg) recovered from Sephadex G-100 (Text-fig. 1). The column (2·5 cm × 30 cm) was equilibrated in 0·01 m-ammonium acetate, pH 5·6, eluted initially with the same buffer and then with increasing concentrations of NaCl (0·10-0·5 mol/l, represented by the arrows). A flow rate of 30 ml/h was used and 10 ml fractions were collected. Hatched area, lactogenic activity; open circles, ultraviolet absorption (E) at 280 nm. A, B, C. Three resolved peaks of lactogenic activity; oPL, ovine placental lactogen.

As the protein in peak B possessed the highest specific activity, this material was re-run on a further column of Sephadex G-100. As can be seen in Text-fig. 3, a peak of lactogenic activity coincided with one of the three peaks of ultraviolet absorption. Fractions 16–32 were pooled, dialysed and freeze-dried to give 4 mg oPL as an off-white powder.

Polyacrylamide gel electrophoresis

Plate 1, fig. 1 shows the results of electrophoresis of samples from various stages in the purification of oPL. Purified oPL was a cationic protein with four minor components
Text-fig. 3. Gel filtration on a column (1-4 cm × 81 cm) of Sephadex G-100 of material (13-3 mg) recovered after carboxymethylcellulose ion-exchange chromatography (Text-fig. 2). The elution buffer was 0-1 M-ammonium bicarbonate, pH 8-4, at a flow rate of 9 ml/h; 4 ml fractions were collected. Hatched area, lactogenic activity; O, ultraviolet absorption (E) at 280 nm; oPL, ovine placental lactogen.

which migrated towards the anode. It was more basic than ovine GH or prolactin, hPL, or human or bovine GH under the conditions used, although the mobility of oPL was close to that of ovine and bovine GH. When electrophoresis was carried out using longer tubes (0-3 cm × 20 cm) of polyacrylamide, the major component of oPL was resolved into two parts (Text-fig. 4). These two major bands and the four minor components all possessed lactogenic activity in the receptor assay (Text-fig. 4).

Text-fig. 4. Distribution of lactogenic activity after polyacrylamide gel electrophoresis of a purified preparation of ovine placental lactogen (oPL, 100 µg) in tubes (0-3 cm × 20 cm) containing 7-5% acrylamide, pH 8-9. Also shown is a diagrammatic representation of the proteins in an adjacent gel stained with Coomassie Blue.

**Isoelectric focusing**

The distribution of human GH-like activity obtained when a preparation of oPL was submitted to isoelectric focusing on polyacrylamide gel is shown in Text-fig. 5. An almost
linear pH gradient was achieved along the length of the gel, from which the isoelectric point of oPL was determined as 8.2–8.4. This corresponds to the presence of oPL concentrated in gel slice number five.

**Molecular weight determination**

Gel exclusion chromatography on a column of Sephadex G-100 was used to estimate the molecular weight of oPL. A molecular weight of approximately 24 000 was obtained by this method (Text-fig. 6), consistent with the observation that the elution volumes of oPL and ¹²⁵I-labelled ovine prolactin were almost identical.
Lactogenic and growth-hormone-like activities of purified oPL

Displacement curves for purified oPL and ovine prolactin in the rabbit mammary gland radioreceptor assay are shown in Text-fig. 7. The two curves are parallel and almost superimposable, indicating that oPL competed for the prolactin receptor. The specific activity of the oPL preparation was 1 mg ovine prolactin/mg protein, which represents a 500-fold purification relative to the specific activity of the initial NH₄HCO₃ extract.

On administration of 150 µg ovine prolactin or oPL into the nipples of mid-pregnant rabbits, secretory activity was stimulated, as revealed by lobulo-alveolar development and the presence of colloid secretions within the alveolar lumina. The diluent had no lactogenic effect.

The displacement curves for oPL, human and ovine GH, ovine prolactin and hPL in the radioreceptor assay for human GH-like activity are shown in Text-fig. 8. Ovine placental lactogen inhibited the binding of ¹²⁵I-labelled human GH in a parallel manner to standard human GH. In this assay system, the other standards, ovine GH, prolactin and hPL, were less active than either human GH or oPL. From the displacement curve it was calculated that our oPL possessed an activity equivalent to 80% of standard human GH, i.e. 1.3 i.u. human GH/mg protein.

Immunological properties of oPL

Anti-oPL serum cross-reacted only with oPL, giving a dense immunoprecipitate (Pl. 2, fig. 2). More discrete immunoprecipitates were obtained when the agar gel was made up in 0.9% (w/v) saline rather than in the conventional sodium barbitone buffer at pH 8.6. The
apparent immunological homogeneity of the oPL preparation was confirmed in the immunoelectrophoresis experiment (Pl. 2, fig. 3). Furthermore, there was no evidence of serum proteins in our preparation.

DISCUSSION

The purification of oPL has been reported by other workers (Handwerger et al. 1974; Hurley et al. 1975; Martal & Djiane, 1975; Chan et al. 1976; Fellows et al. 1976). Our method of purification differs in several respects, as do some of the biochemical properties of our oPL. Our initial extraction of placental tissue was with NH₄HCO₃, similar to the method used by Chan et al. (1976) and Fellows et al. (1976). The specific activity of our product was 2 × 10⁻³ mg oPL/mg protein, compared with the values of 1 × 10⁻³, 9 × 10⁻³ and 6 × 10⁻⁴ mg oPL/mg protein obtained by Chan et al. (1976), Fellows et al. (1976) and Martal & Djiane (1975) respectively.

Methods for the subsequent chromatographic purification of oPL were chosen so as to achieve a high percentage recovery of lactogenic activity using the minimum number of column procedures. Large quantities of non-lactogenic protein were eliminated by use of a Sephadex G-100 column (92% protein removed) with subsequent fractionation on the carboxymethylcellulose column (99% protein discarded). After these two column procedures, the specific activity of the active fraction had increased to 0·86 mg ovine prolactin/mg protein. After the final Sephadex G-100 chromatography step, the specific activity of the oPL obtained was 1 mg ovine prolactin/mg protein (based on a specific activity for ovine prolactin of 24·3 i.u./mg), which is close to the value reported by Chan et al. (1976; 0·98 mg ovine prolactin/mg protein). The material isolated by Martal & Djiane (1975) was
of a lower specific activity (0-6 mg ovine prolactin/mg protein, using a prolactin standard of 24 i.u./mg).

Activity in a radioreceptor assay does not necessarily mean that the protein under study is biologically active. The lactogenic activity of our preparation of oPL was confirmed by its ability to stimulate secretory activity in the mammary gland of the 12-day pregnant rabbit.

In the GH radioreceptor assay, our preparation of oPL possessed a specific activity of 0-8 mg human GH/mg protein using human GH (1-6 i.u./mg) as standard; this figure is close to that reported by Chan et al. (1976; 0-84 mg human GH/mg protein using human GH with a biological activity of 1-9 i.u./mg). Ovine placental lactogen prepared by Handwerger et al. (1974) was only 20% as active as human GH in the radioreceptor assay. The reason for this low GH-like activity is not clear.

The electrophoretic mobility of oPL relative to that of other standard proteins is similar to that described by Martal & Djiane (1975), Chan et al. (1976) and Fellows et al. (1976). Whereas the preparation described by Martal & Djiane (1975) and Fellows et al. (1976) appears to migrate as a single band, the behaviour of our material and that described by Chan et al. (1976) is more complex. Under alkaline conditions, the latter preparation migrated as two distinct bands together with a faster running minor component. However, only one of the major bands possessed lactogenic activity in the receptor assay. Our preparation of oPL was resolved into one major component and four minor species, all of which were shown to be active in the mammary gland receptor assay. Furthermore, the major component could be further resolved into a doublet after electrophoresis in long tubes containing 7-5% acrylamide gel. The relationship between these two compounds and those resolved by Chan et al. (1976) is unknown. We consider that the electrophoretic microheterogeneity of our preparation may be due to deamidation of oPL, a modification which occurs to many purified hormone preparations (Sherwood, 1967) and is exemplified by Pl. 1, fig. 1.

There is a wide discrepancy between the isoelectric points reported for oPL. Our figure of 8-2–8-4 is near the value of 8-8 found by Chan et al. (1976) but differs markedly from the values of 7-2 and 6-7 reported by Martal & Djiane (1975) and Fellows et al. (1976) respectively. Such a variation would be consistent with the products of the latter two groups of workers being degraded through deamidation. An estimate of 24 000 for the molecular weight of oPL is close to the values reported by Martal & Djiane (1975; 20 000–22 000), Chan et al. (1976; 22 000) and Fellows et al. (1976; 23 000).

That oPL was free from serum protein contaminants was confirmed by immunodiffusion experiments. Contrary to our findings and also to those of Martal & Djiane (1975), Handwerger et al. (1974) showed that their anti-oPL serum cross-reacted with ovine GH. Further evidence for the purity of our anti-oPL serum is that with immunocytochemical techniques, there was specific localization of oPL in the trophoblastic cells of the sheep placenta and, furthermore, there was no reaction with slices of sheep anterior pituitary tissue (W. B. Watkins and S. Reddy, unpublished results).

This study was financed by a grant from the Medical Research Council of New Zealand.

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Purification of ovine placental lactogen


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**DESCRIPTION OF PLATES**

**PLATE 1**

Fig. 1. Electrophoresis, in tubes (0.5 cm × 81 cm) of 7.5% polyacrylamide, of lactogenic material from (a) a large (10 cm × 100 cm) Sephadex G-100 column; (b) a carboxymethylcellulose ion-exchange column (peak B; see p. 63); (c) a small (1.4 cm × 81 cm) Sephadex G-100 column. The mobility of ovine placental lactogen in (c) is compared with that of other lactogenic and GH-like proteins: (d) ovine GH; (e) ovine prolactin; (f) human placental lactogen; (g) human GH; (h) bovine GH. All proteins were used at a concentration of 75 µg/gel.

**PLATE 2**

Fig. 2. Microimmunodiffusion on 1% agar in 0.9% (w/v) saline of 5 µl anti-ovine placental lactogen serum (centre well) against (1) ovine placental lactogen; (2) ovine GH; (3) ovine prolactin; (4) human placental lactogen; (5) human GH; (6) normal sheep serum (5 µl). All hormones were at a concentration of 5 µg in a volume of 5 µl.

Fig. 3. Microimmunoelectrophoresis of 2 µl sheep serum (wells 1 and 3) and 4 µg ovine placental lactogen in a volume of 4 µl (well 2). Anti-sheep serum and anti-ovine placental lactogen (100 µl) were placed in troughs A and B respectively.