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Isolation of Choriomammotropin from the Ovine Placentome

C. M. Leach Harper, V. L. Ford and G. D. Thorburn

Department of Physiology, Monash University, Clayton, Vic. 3168.

Abstract

Placentomes, taken from ewes at 101–141 days gestation, were homogenized and subjected to differential and density gradient centrifugation. In the presence or absence of protease inhibitors, choriomammotropin (ovine chorionic somatomammotropin, oCS) was detected by radioimmunoassay, and banded at two positions in 30–60% w/v sucrose gradients. The first band (soluble form) eluted in the sample zone of the gradient and contained 33±6·1% (mean±s.e.m., $n = 10$) of the total oCS present in the gradient. The remainder (48±7·6%) of the oCS (particulate form) sedimented to 37·5–43% w/v sucrose. Both these bands became sharper with decreasing protein loaded onto the gradients. ^{125}I-oCS, pre-equilibrated with a placentome preparation, eluted in the same position as the first band of endogenous oCS. When loaded onto a second gradient the second band eluted at 38–41% w/v sucrose, but after being subjected to ultrasonic disruption or treatment with Triton X-100 this oCS eluted in the sample zone. There was no correlation between gestational age of the ewe and the percentage oCS in either the soluble or particulate form, the concentration of oCS, or enrichment by protein.

We have shown that following homogenization, differential and density gradient centrifugation, oCS exists in two forms. We suggest that the particulate form of the hormone is contained within the electron-dense granules of placentome cells. The soluble form may represent oCS in the process of secretion and hormone which has leached out of the granules during homogenization and centrifugation of the placentomes.

Introduction

Choriomammotropin (ovine chorionic somatomammotropin, oCS) has previously been identified (Forsyth 1973; Fellows *et al.* 1974), purified and characterized (Martal and Djiane 1975; Chan *et al.* 1976; Reddy and Watkins 1978*a*). oCS has been localized by immunocytology in both the binucleate and uninucleate cells of the ovine placentome (Martal *et al.* 1977; Reddy and Watkins 1978*a*; Watkins and Reddy 1980). Recently Carnegie *et al.* (1982) have shown that oCS is confined to the cytoplasm of specific uninucleate chorionic cells in close association with lipid droplets and is not present in the binucleate cells. In contrast, Wooding (1981) has localized oCS exclusively in binucleate cell granules, the Golgi region of the fetal chorionic epithelium and the contiguous syncytial layer of placentomes. The binucleate cells of the ovine placentome are profusely granulated (Wimsatt 1950), while there is no clear indication of whether or not the uninucleate cells contain electron-dense vesicles. Similar granules are observed in many cells which synthesize protein hormones (Costoff and McShan 1969; Parry *et al.* 1979). It is generally accepted that these small electron-dense granules are 'packages' of protein hormones (Parry *et al.* 1979). In preliminary studies, Taylor (1980) observed two bands of oCS from a placental homogenate after centrifugation on a linear 15–55% w/v sucrose gradient. The

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present data support the concept that oCS exists in the placentome in a particulate form, presumably in secretory granules. A preliminary report of these results was presented to the Australian Society for Reproductive Biology, 1983.

Materials and Methods

Ten Merino × Border Leicester ewes were killed by pentobarbitone injection at 101–141 days of gestation. For each ewe, the uterus was excised, and seven or eight placentomes selected at random from different parts of the placenta were immediately removed from the uterus and placed in ice-cold 0·05 M phosphate-buffered saline, pH 7·4 (PBS).

Homogenization and Primary Fractionation of Ovine Placentome

All manipulations were performed at 4°C. The placentomes were decapsulated, the haemophagous zone removed and the remaining tissue coarsely minced with scissors before homogenization in PBS (1 : 5, w : v) in a Potter–Elvehjem homogenizer (Thomas, Philadelphia, PA, U.S.A.; No. C44491, 0·177 mm clearance). The tissue used in these studies included both maternal and fetal tissue, the main cell types being uninucleate, binucleate and syncytial cells. We have called this mixture of cells placentome cells. The homogenizer was operated by hand and the tissue was subjected to 10 strokes of the pestle. According to Willcox (1979), who used the condition of the nuclei as an indicator of gross organelle damage, this procedure causes extensive tissue disruption without damaging the nuclei. The homogenate was centrifuged at 1500*g* for 20 min in a Sorvall RC-5B centrifuge. The supernatant was decanted and further centrifuged at 5000*g* for 30 min in the same centrifuge. The resultant pellet was resuspended in 0·25 M sucrose–10 mM HEPES, pH 7·4 (resuspension medium). An amount of approximately 1 g wet weight of the decapsulated placentome was resuspended in 2 ml of the resuspension medium. This suspension was homogenized by hand with three strokes of the Potter-Elvehjem homogenizer and immediately loaded onto sucrose gradients.

A second homogenate was prepared from ewe 9 (9b in Table 1) in the presence of protease inhibitors (0·05 M ethylenediaminetetra-acetic acid, 0·1 M 6-aminohexanoic acid, 5 mM benzamidine HCl and 0·5 mM phenylmethylsulfonyl fluoride).

Density-gradient Centrifugation

Linear 30–60% w/v sucrose gradients were formed in seven 5-ml steps over 1 ml 65% w/v sucrose, and allowed to diffuse for 24 h at 4°C, forming linear gradients. All sucrose solutions were buffered by 10 mM HEPES, pH 7·4. A 2·5–3 ml sample, containing 2·0–6·4 mg protein, was loaded onto all gradients except those from ewe 10. In order to obtain sharper peaks of oCS within the sucrose gradient, various amounts of homogenate, and thus protein, were loaded onto the gradients of ewe 10. The homogenate, obtained by the above procedure, was diluted in resuspension medium and 0·49, 0·98 and 1·43 mg protein samples were loaded onto gradients.

All gradients were centrifuged at 90 000g_{av} for 150 min in a SW27 Rotor in a Beckman L8-65B ultracentrifuge. The gradients were eluted in 1-ml fractions from the base of the gradient through a tube attached to a peristaltic pump. The fractions were frozen and stored at −20°C until assay.

To determine the migration of soluble oCS in the gradients, a second placentome preparation from ewe 7 was prepared, as described above, and equilibrated with ^{125}I-oCS (200 000 cpm) for 1 h at room temperature (22°C). This mixture was then loaded onto 30–60% w/v linear sucrose gradients and centrifuged and eluted, as described above. The resultant sample zone was pooled, diluted with 10 mM HEPES, pH 7·4, to 8·4% w/v sucrose and loaded onto linear 10–30% w/v sucrose gradients. These gradients were formed over 1-ml 35% w/v sucrose in five 7-ml steps and stored at 4°C for 24 h before use. The 10–30% w/v sucrose gradients were centrifuged and eluted as described above for the 30–60% w/v sucrose gradients. The ^{125}I-oCS content of each fraction was determined in an LKB ultragamma counter.

To determine the stability of the particulate form of oCS, fractions from gradients of ewe 1 containing this form of oCS were immediately either subjected to ultrasonic disruption for 5 min or treated with Triton X-100 (Waters and Friesen 1979), or prepared in the presence of protease inhibitors (ewe 9b). These preparations were then diluted with 10 mM HEPES, pH 7·4, to 8·4% w/v sucrose and loaded onto 30–60% w/v linear sucrose gradients. The gradients were centrifuged and eluted as described above.

Sucrose concentrations (w/v) were measured by refractometry, using an Atago (Japan) refractometer. Protein concentration was measured by the Coomassie Blue method of Bradford (1976), with a protein dye binding kit purchased from Bio-Rad Laboratories (Calif., U.S.A.).

Ovine Choriomammotropin Assay

Ovine choriomammotropin was assayed in each gradient fraction by the method of Chan *et al.* (1978). The oCS standard was prepared by the method of Chan *et al.* (1978) and the iodination of oCS was performed using the lactoperoxidase method of Thorell and Johansson (1971). The oCS standard and antisera was a generous gift from Dr M. J. Waters, the antisera being raised against purified oCS in goats. Cross-reaction of the antiserum with ovine pituitary hormones (luteinizing hormone, follicle stimulating hormone, prolactin, growth hormone and thyroid stimulating hormone) was <1%. The intra- and inter-assay coefficients of variation were 3·4 and 20·1% respectively, and all fractions from a gradient were assayed in a single assay. The sucrose present in the gradient fractions did not interfere with the assay. The correlation coefficient of the relationship between the amount of oCS added to 30% w/v sucrose and that measured was 0·990 ($P < 0·001$), and the slope of the regression line was 0·853. The gradient fractions diluted parallel to the standard curve and were assayed in the dilution range of neat to 1 : 10 000, which gave a sensitivity range of 4 ng/ml to 200 μg/ml oCS.

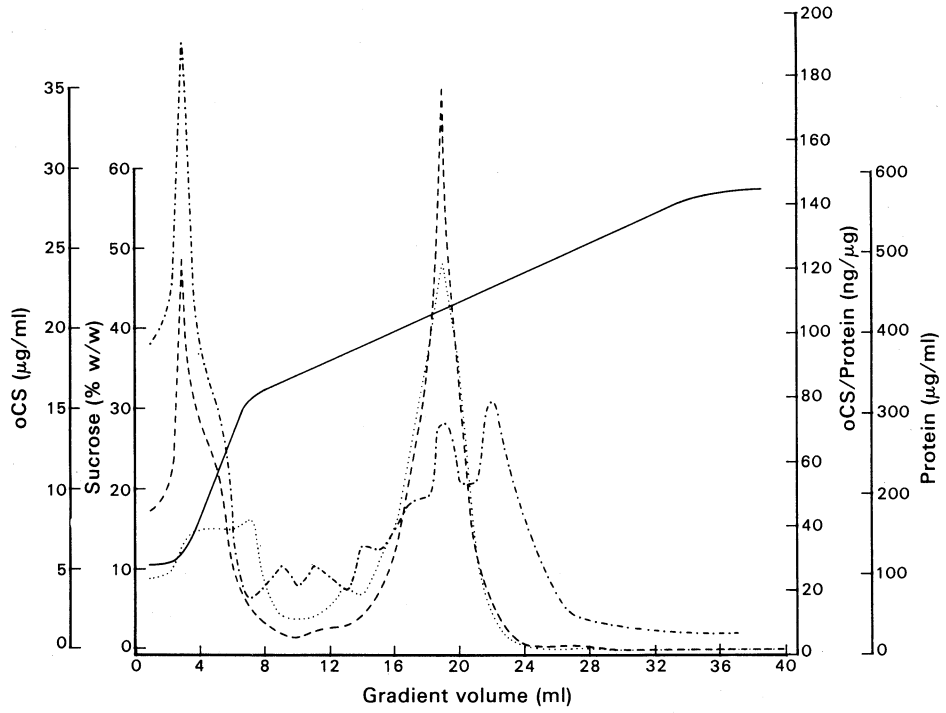

Fig. 1. Density gradient centrifugation of a fraction of ovine placental homogenate from ewe 1, on a linear 30–60% w/v sucrose gradient. ——— Sucrose concentration. ····· Protein. - - - - oCS. -·-·-·- oCS/protein.

Results

The distribution of immunoreactive oCS on a linear 30–60% w/v sucrose gradient is shown in Fig. 1. This profile is representative of the profiles obtained from all ewes. There were two regions of oCS in the gradient profile. One band eluted in the sample zone at the top of the gradient and contained 80 μg oCS, which was 42% of the total oCS. The

peak concentration of oCS in the sample zone was 24·5 μg/ml and eluted at 12·4% w/w sucrose. A second band of immunoreactive oCS entered the gradient, eluted at 38·4–45·1% w/w sucrose and contained 107 μg oCS, which was 56% of the total oCS. The peak concentration of oCS in this second band was 34 μg/ml and eluted at 42·5% w/w sucrose. In order for the second band of oCS to elute in this position it must be associated with some particulate fraction of a placentome cell. Thus this band of oCS was nominated the particulate form of the hormone. The oCS which did not enter the gradient was nominated the soluble form of the hormone. The elution profile of protein for this gradient is also shown in Fig. 1. Two bands of protein were observed, and these were in the same regions of the gradient as the oCS. There was 35% of the protein in the sample region and 59% of the total protein in the region of the particulate oCS. The oCS to protein ratio (ng/μg) for these two regions was 70·7 and 49·7, with peak values of 189·0 and 70·8 for the sample and particulate regions respectively. Thus there was a mean enrichment of oCS, by protein, of 1·3-fold in the sample zone of the gradient and 0·8-fold for the oCS which sedimented into the gradient. A plot of the oCS to protein ratio for all fractions of the gradient is shown in Fig. 1 and details of this profile are in Table 1.

Table 1. Distribution of oCS and protein (pr.) in linear 30–60% w/v sucrose density gradients
Total oCS and protein were determined by the addition of the oCS and protein content of all fractions of the gradient. The soluble oCS is defined as that oCS which remained in the sample zone of the gradient and the particulate oCS as that which sedimented into the gradient. n.d., not determined

Ewe No.	Gestation age (days)	Total oCS		Soluble oCS		Particulate oCS	
		oCS (μg)	oCS/pr. (ng/μg)	oCS (%)	oCS/pr. (ng/μg)	oCS (%)	oCS/pr. (ng/μg)
1	101	190	58·2	42	70·7	56	49·7
2	102	48	22·0	44	42·5	49	20·1
3	110	394	61·5	85	136·6	14	17·1
4	124	175	44·9	44	149·8	55	46·0
5	138	257	44·7	27	40·4	71	47·8
6	139	166	81·6	42	108·7	56	66·1
7	141	118	37·5	46	70·1	46	29·0
8	141	62	23·5	27	16·4	70	34·0
9a	120	51	n.d.	33	n.d.	63	n.d.
9b[A]		50	n.d.	32	n.d.	66	n.d.

[A] Homogenate prepared in the presence of protease inhibitors, in contrast to all other preparations.

The placentomes of ewes 1–9a were manipulated in an identical manner, and those from ewe 9b were homogenized in the presence of protease inhibitors. The addition of the protease inhibitors had no effect on the distribution of soluble and particulate oCS isolated from placentome homogenates (Table 1).

In all gradients the oCS was present in the two regions described above. The percentages of total oCS in the soluble and particulate forms in all gradients are shown in Table 1. There was a mean±s.e.m. of 33±6·1% of the total oCS in the soluble form and 48±7·6% of the oCS in the particulate form. The enrichment of soluble oCS in the gradient ranged from 0·9 to 3·3-fold and of particulate oCS 0·3–1·6-fold.

In order to achieve sharper peaks of the soluble and particulate forms of oCS, varying amounts of the placentome preparation from ewe 10 were loaded onto 30–60% w/v sucrose gradients. The elution position of the soluble oCS in the sample zone was independent of the amount of protein loaded onto the gradient, although with decreasing protein load on the gradient the soluble peak became sharper (Fig. 2). The maximum concentration of particulate oCS eluted at 42% w/w sucrose in all cases and with decreasing protein load,

the particulate oCS peak became sharper. The concentration of soluble and particulate oCS and protein diluted in parallel to the dilution of the preparation with resuspension medium. The percentage oCS and protein in both peaks remained constant.

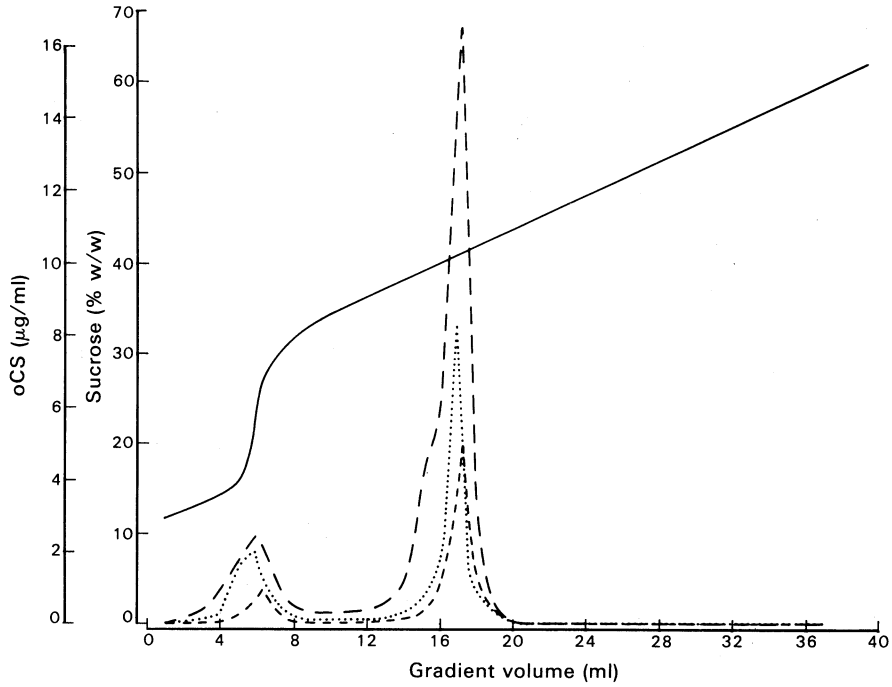

Fig. 2. The effect of decreasing protein load on the migration of oCS into linear 30–60% w/v sucrose gradients. Protein load 0·49 mg (– – – –); 0·98 mg (· · · · ·); 1·43 mg (——).

To determine whether or not the band of oCS in the sample zone of the 30–60% w/v sucrose gradients was 'soluble' hormone and where free oCS would elute in these gradients, ^{125}I-oCS was loaded onto sucrose density gradients. The ^{125}I-oCS was first equilibrated with a placentome preparation for 1 h at 22°C to allow interaction between the free hormone and the particulate matter and then loaded onto a 30–60% w/v linear sucrose gradient, centrifuged and eluted as described above. The ^{125}I-oCS eluted in the sample zone, as shown in Fig. 3, and did not migrate into the gradient. One of the bands of endogenous oCS (Fig. 1) eluted at this identical position in the sample zone. The ^{125}I-oCS fractions were then loaded onto linear 10–30% w/v sucrose gradients. The ^{125}I-oCS eluted in the same position in the sample zone of this gradient, as shown in Fig. 3.

The nature of the particulate matter containing the oCS was investigated by manipulation of the fractions from sucrose gradient containing the particulate oCS. The particulate oCS from the placentome homogenate of ewe 9a, prepared in the absence of protease inhibitors, and the particulate oCS from ewe 9b, prepared in the presence of protease inhibitors, re-sedimented to 38–41% w/w sucrose when loaded onto a 30–60% w/v linear sucrose gradient. No free oCS was formed from either preparation of particulate oCS. In contrast, after ultrasonic disruption or treatment with Triton X-100 the particulate form of oCS from ewe 1 did not sediment into 30–60% w/v sucrose gradients but remained in the sample zone. This indicates that the organelle containing the oCS had been destroyed and the oCS was released to sediment as free oCS in the gradient.

The effect of gestational age on all the parameters discussed above is shown in Table 1. The total oCS present in the gradients ranged from 48 to 394 μg oCS, which was 22·0–81·6 ng oCS/μg protein. There was no correlation between gestational age and the total oCS, total protein or oCS to protein ratio. Generally, a greater percentage of oCS was in the particulate form, rather than the soluble form, except for ewe 3 where only 14% of the

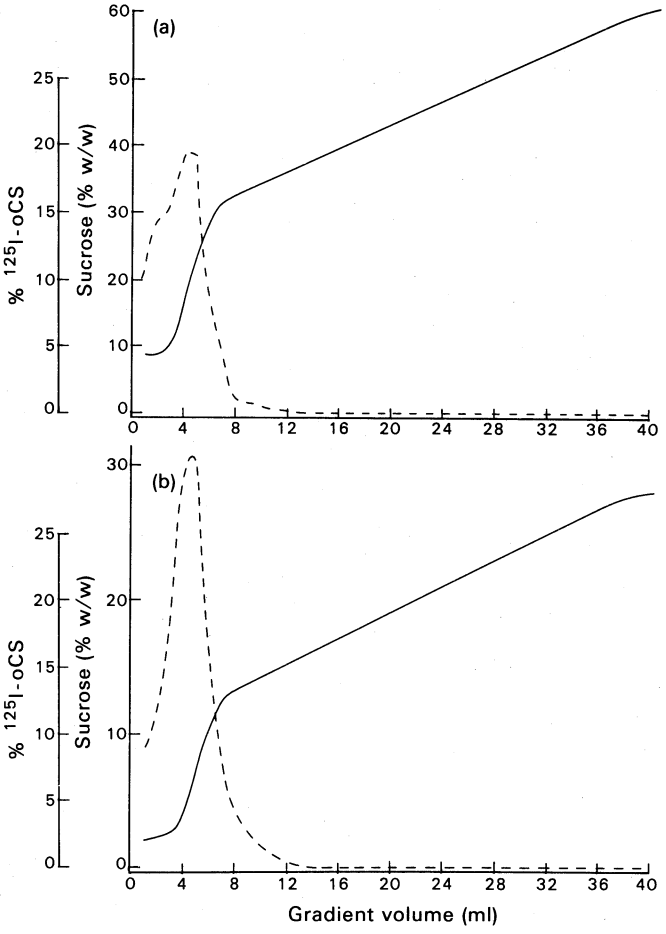

Fig. 3. Sedimentation of ^{125}I-oCS on linear 30–60% w/v (*a*) and 10–30% w/v (*b*) sucrose density gradients. ^{125}I-oCS was calculated from the amount of radioactivity loaded onto the gradient

oCS was in the particulate form. The oCS was enriched in the sample zone of almost all gradients. There was no correlation between gestational age and the percentage oCS or oCS to protein ratio for either the soluble or particulate forms of the hormone or the enrichment of these forms of oCS in the gradient.

Discussion

We have shown that there are two forms of choriomammotropin in the ovine placenta *in vitro*. One form of the hormone does not sediment from the sample zone of a linear 30–60% w/v sucrose gradient. Evidence indicating that this was free, soluble oCS was

obtained by characterizing the sedimentation behaviour of ^{125}I-oCS, pre-equilibrated with a preparation of ovine placentome. The ^{125}I-oCS and a portion of the endogenous oCS banded at an identical position in the sample zone of the gradient. Furthermore the ^{125}I-oCS banded in the same position of the sample zone of both 30–60% w/v and 10–30% w/v sucrose gradients, again indicating that the endogenous 'soluble' hormone was not attached to any particulate matter. Homogenization of the placentome in the presence of protease inhibitors did not influence the relative concentration of soluble and particulate oCS, indicating that these forms of the hormone are not a result of protease degradation of particulate oCS. The presence of free oCS is probably due to the homogenization and centrifugation of the placentome.

The second form of oCS reported in this study migrated to 37·5–43% w/w sucrose (density 1·1638–1·1920 g cm^{-3}) in a linear 30–60% w/v sucrose gradient. To migrate to this density within the sucrose gradient, this second form of oCS must be bound to a membrane or contained within an organelle or secretory granule of the placentome cell. Our results demonstrate that the subcellular structure containing the particulate oCS is stable in sucrose. The particulate form of oCS re-sedimented to 38–41% w/w sucrose on a second 30–60% w/v sucrose gradient and did not dissociate into soluble and particulate forms of the hormone. This suggests that the presence of both forms of oCS in the homogenate is not a function of an equilibrium between two states, but a function of containment of a portion of oCS within a stable subcellular structure. The structure containing particulate oCS was destroyed by ultrasonic disruption or treatment with Triton X-100, which are known to disrupt secretory granules. Upon destruction of this structure the oCS sedimented as soluble, free oCS on a sucrose gradient, indicating that it was not attached to a membrane.

In the preliminary studies of Taylor (1980), two bands of oCS were obtained after centrifugation of a placental homogenate on a linear 15–55% sucrose gradient. One band of oCS remained in the sample zone of the gradient while the other band eluted to 38–45% w/w sucrose, with the peak fraction at 42% w/w sucrose. These two forms of oCS were observed in our experiments and sedimented to similar densities on linear 30–60% w/v sucrose gradients.

Secretory granules containing protein hormones have been isolated using differential and density gradient centrifugation techniques (Costoff and McShan 1969). Immunoreactive gastrin sediments to 39·5% w/w sucrose (Trotman *et al.* 1976) and the granules from rat anterior pituitary glands separate on 6–45% w/v sucrose gradients. The diameter of reported electron dense granules containing protein hormones ranges from 85–900 nm, for different cell types (Costoff and McShan 1969). The granules of the ovine corpus luteum have a mean diameter of 200 nm (Gemmell *et al.* 1974). The exact diameter of the granules in the ovine placentae has not been determined, although the granules would appear to be of a similar size to those of the ovine corpus luteum (Wooding 1981). Thus the electron dense granules of the ovine placental cells might be expected to sediment into the 30–60% w/v sucrose gradients used in these experiments.

The ratio of oCS to protein in the placental homogenate was not greatly enriched by the separation of the soluble and particulate forms of the hormone by density-gradient centrifugation. In all placental homogenates studied, the particulate oCS sedimented to the same density as the organelles, as indicated by the protein concentration. Thus, although there is clear separation of the oCS forms, the procedure outlined in this paper is not an appropriate method for isolating the particulate oCS from the remaining organelles of the placentome cells.

This paper provides evidence that oCS is present in a particulate fraction of the placentome cells. We propose that oCS is stored within the electron dense granules of these cells, while we cannot indicate which cell type/s contain these granules.

Acknowledgments

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