Proteins in the uterine secretions of the cow

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Summary. Cows of normal reproductive history were treated with progesterone for periods of 2–3 months. The uterine secretions yielded 5 major fractions of macromolecular components. Two of the fractions comprised serum proteins. In the other 3 fractions at least 9 non-serum proteins were observed, 7 in one fraction, but in only small amounts. Of the remaining 2 non-serum proteins one is an acid phosphatase recoverable in small amounts, and the other is lactoferrin and is the major non-serum protein present in the uterine secretions of progesterone-treated cows.

Introduction

The uterine secretion, constituting as it does the environment of the early embryo, is likely to be of importance in mammalian reproduction, particularly in ruminants in which the processes of implantation and placentation are relatively slow (Milton, Berry & Butler, 1951; Greenstein, Murray & Foley, 1958). The first clear confirmation of this importance stems from the embryo transplant work of Rowson & Moor (1966) and Rowson, Lawson, Moor & Baker (1972), who found a rather stringent requirement for synchrony between donor and recipient. This implies that the uterine environment is changing quite rapidly with time and furthermore that such changes in composition are vitally concerned with the survival of the embryo.

The information we have at present on the composition of the mammalian uterine secretion is extensive but somewhat superficial (man: Beier, Petry & Kühnel, 1970; Wolf & Mastroianni, 1975; Roberts, Parker & Henderson, 1976a; rat: Kunitake, Nakamura, Wells & Moyer, 1965; Perrine, 1967; Dupont-Maïresse & Galand, 1975; Umpathysivam & Breed, 1976; baboon: Peplow, Breed, Jones & Eckstein, 1973; mink, dog, armadillo, black bear: Daniel & Krishnan, 1969; seal: Daniel, 1972; golden hamster: Noske & Daniel, 1974; sheep: Wales, 1973; cow: Roberts & Parker, 1974a, b; Roberts, Parker & Symonds, 1976b; Laster, 1977; mare: Zavy, Bazer & Sharp, 1976; various marsupials: Renfree, 1973, 1975). By and large this work serves mainly to emphasize the extraordinary biochemical diversity which appears to exist among mammalian reproductive systems. There are two species which have been studied more thoroughly and from which uterine proteins (i.e. proteins not also found in the serum of the same animal) have been isolated. These are the rabbit and the pig, from which, respectively, uteroglobin (Beier, 1968, 1974; Krishnan & Daniel, 1968) and a phosphatase (Murray, Bazer, Wallace & Warnick, 1972; Chen, Bazer, Cetorelli, Pollard & Roberts, 1973; Schlosnagel, Bazer, Tsibris & Roberts, 1974) have been purified. Uteroglobin, a glycoprotein which binds progesterone (Beato & Baier, 1975) is a major component of rabbit uterine contents. The porcine acid phosphatase, a glycoprotein, is likewise a major component of the uterine secretions; it is secreted under the influence of exogenous progesterone, contains iron and is purple in colour. Despite much search, in no other species has anything quite like these proteins, certainly not in the amounts encountered in the rabbit and the pig, been found. A possible exception is the mare which, according to one report, produces a protein similar to that of the pig (Zavy *et al.*, 1976).

* Died on 7 July 1978.

The exploratory work of Roberts & Parker (1974a) and Laster (1977) has demonstrated that uterine proteins are present in the cow. Initially the uterine washings used by Roberts & Parker (1974a) provided a source of uterine protein but it rapidly became apparent that, with an average yield of 15 mg total protein per uterine washing, of which not more than 300 µg were the materials sought, this source was inadequate. Roberts & Parker (1976) gave immunochemical evidence that protein of uterine origin may be found in allantoic and amniotic fluid. This is a far more copious source and much work has been done in an endeavour to isolate the uterine proteins from it. The fluids do have one protein in common: acid phosphatase found in fetal fluids has the same pH optimum and isoelectric point (9.7) as the enzyme later found in bovine uterine fluid (see below). However, there are also very large amounts of protein of non-uterine origin in the fetal fluids.

Harrison, Heap, Horton & Poyser (1972) showed that surgical separation of the uterus and the ovaries in the ewe caused the animal to become pseudopregnant and fluid to accumulate in the uterus. A preliminary electrophoretic examination of a specimen of this fluid at pH 3.5 showed prominent protein bands migrating as do the uterine proteins reported by Roberts & Parker (1974a). A similar result is obtained with intact animals treated with large amounts of exogenous progesterone, and the uterine fluid of such animals was therefore investigated more extensively.

Materials and Methods

Recovery of uterine secretions. Cows of normal reproductive history were removed from the Institute herd and observed for oestrus. They were then given progesterone, 300 mg subcutaneously in oil, daily for 2–3 months. At slaughter the uteri were exposed as rapidly as possible, clamped at the utero-tubal junctions, ligated just cranial to the internal os and excised. The uterine contents were allowed to drain from one of the uterine horns, and/or were rinsed out of this organ with 0.01 M-phosphate buffered saline, pH 7.2, introduced at one utero-tubal junction and recovered, after inversion and gentle massage, from the other.

Preparation of uterine proteins. The uterine contents and washings were centrifuged to remove cellular debris and the supernatant was dialysed against 0.1 M-sodium acetate buffer, pH 5.5. To this solution (150–175 ml) was added a slurry (50 ml) of carboxymethyl cellulose (Whatman CM52) in the same buffer and the resulting mixture was stirred slowly at 4°C overnight. The carboxymethyl cellulose was recovered by filtration and washed with 0.1 M-sodium acetate buffer, pH 5.5, to remove unbound protein. The carboxymethyl cellulose was then resuspended in buffer and poured into a glass column (1.5 × 30 cm; Pharmacia). The column was washed with 2 volumes of buffer, followed by elution of the bound proteins with 1 M-sodium acetate, pH 5.5. The eluted proteins were redialysed against 0.1 M-sodium acetate, pH 5.5, reabsorbed onto carboxymethyl cellulose (Whatman CM52) and eluted using a linear ionic strength gradient between 0.1 and 1.0 M-sodium acetate, pH 5.5. The eluate was monitored at 280 nm and also tested for acid phosphatase activity by the method of Schlosnagel *et al.* (1974). Fractions were pooled according to the elution profile to give 5 major protein fractions (Text-fig. 1).

Electrophoretic methods. Disc gel electrophoresis of the uterine protein fractions was carried out at pH 9.5 (Davis, 1964) and pH 3.5 (Reisfeld, Lewis & Williams, 1962). Protein bands were stained with coomassie blue, 0.3% in 20% trichloroacetic acid, followed by destaining in ethyl alcohol, acetic acid and water (9:2:9 by vol.). Acid phosphatase activity in gels was demonstrated as described by Andrews & Pallavicini (1973).

Preparative isoelectric focusing was carried out using a glycerol concentration gradient (20–60% w/w) between pH 7 and 11.

Gel filtration. Gel filtration of the uterine protein fractions was carried out on columns of Sephadex G200, G100 or G75 (1.5 × 90 cm, Pharmacia) at 4°C; the eluant was 0.3 M-sodium

chloride containing 0.02% (w/v) sodium azide. The protein content of the eluate was measured by u.v. absorption at 280 nm.

Affinity chromatography. An immunoglobulin fraction of rabbit antiserum against whole bovine serum was precipitated with 16% sodium sulphate at room temperature. The precipitated immunoglobulins were washed with 16% (w/v) sodium sulphate, redissolved in 0.2 M-sodium citrate buffer, pH 6.5, and dialysed against the same buffer (2 litres) overnight at 4°C. This immunoglobulin solution was then coupled to Sepharose 4B activated with cyanogen bromide (Porath, Axen & Ernback, 1967). The coupled gel was then packed into a glass column (1.5 × 15 cm) and washed with 3.0 M-sodium thiocyanate to remove unbound protein. The gel was then washed with 0.2 M-Tris-HCl, pH 8.0, before use.

The sample was applied to the affinity column after dialysis against 0.2 M-Tris-HCl, pH 8.0. After washing with buffer the adsorbed proteins were eluted with 3 M-sodium thiocyanate.

Analytical ultracentrifuge experiments. Sedimentation coefficients were determined at three protein concentrations (1, 0.5 and 0.25%) and extrapolated to zero concentration, even though the virial coefficients were small. Equilibrium runs were made in the synthetic boundary cell by the procedure of Chervenka (1970) using 0.15 ml of a 0.5% (w/v) protein solution with 0.4 ml buffer. Silicone oil (0.02 and 0.04 ml) was placed in the solvent and solution channels respectively. Barbiturate buffer, 0.01 M, pH 7.0, in 0.1 M-NaCl was used as solvent throughout. Its density and viscosity were measured at 20°C using a 50 ml pyknometer and an Ostwald viscometer respectively.

Molecular weights. These were determined from plots of $\text{Ln}C$ (C = concentration) against r^2 (r = radius of rotation) from the equilibrium experiment, using Equation 2.10 of Creeth & Pain (1967). The partial specific volume of lactoferrin was taken as 0.722 ml/g protein (Castellino, Fish & Mann, 1970).

Amino acid analyses. These were carried out automatically (LKB 4101 apparatus) on material (~ 1 mg) which had been hydrolysed for 24 h in 6 M-HCl containing 0.1% thioglycollic acid. Cysteine was determined on a separate aliquot after oxidation with performic acid (Partridge & Davis, 1955).

Carbohydrate analyses. Hexoses were determined according to Yemm & Willis (1954); hexosamines according to Randle & Morgan (1955); neuraminic acid by the method of Warren (1959). Samples were also hydrolysed in Dowex 50 (H^+ form) as described by Gibbons (1962), reduced and acetylated (Roberts, 1974) and analysed by gas-liquid chromatography.

Results

The result of the preliminary separation is shown in Text-fig. 1. The yields of protein (mg) in each of the 5 primary fractions from the pooled material from 3 cows were respectively 1–17.9, 2–18.6, 3–14.0, 4–10.0 and 5–15.9. In addition, the yields of fractionated uterine secretions from 3 individual cows were also recorded (Table 1).

Fractions 1 and 2

Immunodiffusion against antiserum to whole bovine serum indicated that both these fractions contained serum protein. Gel filtration on G200 or G100 failed to effect a useful separation, although Fraction 2 did appear to have small amounts of material which behaved electrophoretically like the uterine proteins. Subsequently attempts were made to remove the serum protein on immunoabsorbent columns as described. Only very small amounts of protein were not taken out by the column and this material still reacted with the anti-bovine serum. It migrated electrophoretically as a γ -globulin but was not immunologically identical with IgG, IgM or IgA.

Text-fig. 1. The gradient elution on carboxymethyl cellulose of uterine proteins from progesterone-stimulated cows, showing the 5 fractions. - - - - - Concentration gradient; ----, acid phosphatase.

Table 1. The protein yields (mg) of each fraction obtained from the gradient elution on carboxymethyl cellulose of uterine protein obtained from cows treated with progesterone for 2 or 3 months

Fraction	Cow G285 (2 months)	Cow J134 (2 months)	Cow J200 (3 months)
1	41.68	4.65	15.87
2	11.2	14.27	8.79
3	4.16	4.96	15.34
4	4.12	32.08	2.36
5	3.3	18.65	4.74
Total protein recovered	660	494	592

Text-fig. 2. Gel filtration (G75 Sephadex) of Fraction 3 (see Text-fig. 1) showing the 3 sub-fractions.

PLATE 1

Polyacrylamide gel electrophoresis (pH 3.5) of the 3 sub-fractions obtained (see Text-fig. 2) from cow uterine secretions. S = bovine serum.

(Facing p. 122)

Fraction 3

This fraction contained chiefly protein which migrated electrophoretically (pH 3.5) at the same rate as the uterine proteins detected by Roberts & Parker (1974a). It was further fractionated on Sephadex G75 to yield 3 sub-fractions (Text-fig. 2). The bulk of this fraction (Sub-fraction 3) was of low molecular weight, and contained at least 4 electrophoretic components (Plate 1). One of these was also present as the major component of the fraction immediately preceding it in the G75 elution (Text-fig. 2). In addition 3 electrophoretic components were observed in Sub-fraction 1. None of the 7 proteins observed formed precipitin bands with anti-serum to whole bovine serum.

Text-fig. 3. Equilibrium sedimentation of bovine uterine lactoferrin in the analytical ultracentrifuge. $\text{Ln } C$ (C = Concentration) against r^2 (r = radius of rotation) at time t (120 h 47 min) after reaching running speed. r_b^2 = cell base.

Fraction 4

This fraction contained very little protein, but almost all of the acid phosphatase activity. The major contaminant was Fraction 5, which could be removed by gel filtration on Sephadex G75 when it emerged with the excluded volume, well separated from the enzyme activity which emerged immediately afterwards. This material appeared homogeneous electrophoretically in gels at pH 9.5 and 3.5. Isoelectric focusing isolated all the activity at pH 9.7.

Fraction 5

This fraction consisted of a protein which gave pale brown solutions and was identical immunologically with bovine milk lactoferrin. It was a single component electrophoretically at both pH 9.5 and 3.5, possessing the same electrophoretic mobility as shown by bovine milk lactoferrin. It sedimented as a single component in the ultracentrifuge at pH 7.0, $S_{20w}^0 = 5.62$. The molecular weight deduced from the linear portion of the $\text{Ln } C/r^2$ plot in the equilibrium sedimentation study was 8.1×10^4 . There was some indication of aggregation, as shown by the slight curvature of the $\text{Ln } C/r^2$ plot towards the base of the cell, resulting from the conditions of pressure and concentration at the base of the cell (Text-fig. 3). The sugar and amino-acid composition of the uterine lactoferrin was compared (Tables 2 and 3) with that of a sample of milk lactoferrin analysed simultaneously.

Table 2. Amino acid analysis of the lactoferrin (as mg/100 mg amino acid recovered) from the uterine secretions and milk of the cow

Amino acid	Bovine uterine lactoferrin	Bovine milk lactoferrin
Asparagine	9.85	9.81 (10.4)*
Threonine	4.87	5.07 (4.94)
Serine	5.18	5.13 (5.11)
Glutamine	11.68	11.92 (11.74)
Proline	4.43	4.31 (4.42)
Glycine	4.39	4.31 (4.39)
Alanine	6.74	6.82 (6.91)
Cysteine (S-S)	5.75	4.97 (5.38)
Valine	6.55	6.40 (6.29)
Methionine	0.84	0.74 (0.878)
Isoleucine	2.01	2.21 (2.47)
Leucine	9.88	9.88 (9.88)
Tyrosine	4.73	4.57 (4.44)
Phenylalanine	5.34	5.44 (5.04)
Histidine	1.88	1.81 (1.91)
Lysine	8.84	9.06 (8.61)
Arginine	7.08	7.39 (7.51)
NH ₃	0.61	0.72

* Data from Gordon, Groves & Basch (1963).

Table 3. Carbohydrate analysis of the lactoferrin from the uterine secretions and milk of the cow, expressed as % by weight of total protein

	Uterine lactoferrin (present study)	Milk lactoferrin (present study)	Milk lactoferrin	
			Groves (1960)	Castellino <i>et al.</i> (1970)
Hexose	4.18*	5.614	4.65	5.6
Hexosamine	2.37	2.15	2.25	2.87
Sialic acid	0.19	0.20	0.3	0.4

* Individual hexoses by g.l.c.: fucose, 0.19%, galactose 0.80%, glucose 0.22%, mannose 3.0%.

Discussion

The criterion for recognition of bovine uterine proteins is based on their electrophoretic mobility. For example, Roberts & Parker (1974a) observed that certain uterine proteins migrated more rapidly at pH 3.5 than any serum component and were therefore easily distinguishable. These uterine proteins comprise not more than 2% of the total protein in the uterine secretion (Roberts & Parker, 1974a), the remainder being identical immunologically with serum proteins and probably derived from the serum (Crutchfield & Kulangara, 1973). A total of 9 uterine proteins have been isolated from this source.

In the present limited study, quantitative variations were observed in all fractions obtained from the gradient elution on carboxymethyl cellulose of the uterine secretions from progesterone-stimulated cows (Table 1). There did not appear to be any relationship between the period of progesterone treatment and the amount of protein recovered in each fraction. The data also suggest that there are variations in the response of individual animals to this progesterone treatment. However, 9 uterine proteins were isolated; one, obtained in a very low yield, was an

acid phosphatase, 7 were from one fraction only, while the major protein was found to be identical to bovine milk lactoferrin. The equilibrium studies of this uterine protein revealed a molecular weight of 8.1×10^4 , compared with published values for milk lactoferrin of 8.1×10^4 (Groves, 1960) and 7.7×10^4 (Castellino *et al.*, 1970). Aggregation behaviour has also been reported (Castellino *et al.*, 1970) for a sample of milk lactoferrin, and the sedimentation coefficient obtained for the uterine lactoferrin agrees well with published values for milk lactoferrin (Groves, 1960; Johansson, 1969).

Lactoferrin, deriving its name from the source from which it was originally isolated, is not peculiar to milk but has been detected in many other secretions and tissues, e.g. human endometrial secretions and tissue (Masson, Heremans & Ferin, 1968a; Tourville, Ogra, Lippes & Tomasi, 1970), seminal fluid, cervical mucus, urine, tears, bile and pancreatic juice (Masson, Heremans & Dive, 1966), kidney cells (Masson, Heremans, Schonke & Crabbe, 1968c), intestinal secretions (Lousillier, Gor, Burton & Grabar, 1967) and bronchial secretions and saliva (Masson, Heremans, Prignot & Wauters, 1968b). It has been shown to have bactericidal properties, a function which is based on the ability of lactoferrin to chelate ionizable iron from culture medium (Masson & Heremans, 1967; Masson *et al.*, 1968b; Blanc, 1967; Schade, 1967; Oram & Reiter, 1968) and it is often inferred that it has this function in the secretions in which it is found. Lactoferrin may thus function as a bacteriostat in the bovine uterus.

In human endometrial secretions lactoferrin is secreted during the last part of the menstrual cycle under the stimulus of progesterone (Masson *et al.*, 1968a). In the present study uterine lactoferrin was secreted by cows treated with exogenous progesterone. It thus appears that progesterone is involved in the secretion of lactoferrin in women and cows. However, in the pig, exogenous progesterone does not stimulate synthesis and secretion of lactoferrin by the endometrium, but does stimulate the secretion of the purple acid phosphatase, lysozyme, leucine amino peptidase and cathepsin (B₁, D and E). Lysozyme, which is absent from bovine uterine secretions, is thought to have a bactericidal function in the porcine uterus (Roberts, Bazer, Baldwin & Pollard, 1976c).

The presence of both an acid phosphatase and metalloprotein in the pig and the cow suggests that they are both essential in some way to uterine function. In the pig, the two functions are combined in one protein, while the cow possesses two proteins. It has been postulated that the porcine acid phosphatase plays an important role in the transport of iron to the fetus as a transferrin-like molecule (Chen, Bazer, Gebhardt & Roberts, 1975; Roberts *et al.*, 1976c). A similar role can be postulated for lactoferrin, although we have failed to detect the presence of lactoferrin in either bovine allantoic or amniotic fluid.

We thank Mr P. Harrison and Mrs D. Knight for their technical assistance during this study.

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Received 1 August 1978