Re-examination of the presence of α -lactalbumin in the epididymis of the rat*

M. Hölpert and T. G. Cooper

Max Planck Clinical Research Unit for Reproductive Medicine, and Institute of Reproductive Medicine of the University, Steinfurter Straße 107, D-4400 Münster, Federal Republic of Germany

Summary. Using an assay for α -lactalbumin in which galactosyltransferase activity was stabilized and a tissue phosphatase inhibitor was present, no evidence was found for α -lactalbumin-like activity in rat epididymal tissue, epididymal fluids or medium from cultured epididymal epithelial cells with either glucose or *N*-acetylglucosamine as acceptor. However, when assay conditions were suboptimal, apparent transfer of radioactivity to both acceptors could be demonstrated in the epididymis and other tissues. In these assays the amount of α -lactalbumin registered was linearly correlated to the extent of stimulation of α -lactalbumin added exogenously to tissue extracts as internal standards. When rete testis fluid from rats was used as source of galactosyltransferase under suboptimal conditions, no transfer to glucose was demonstrable in epididymal fluid and an apparent decreased transfer to *N*-acetylglucosamine could be explained by increases in (pyro)phosphatase activity. Putative α -lactalbumin activity in the epididymis may be an artefact of unoptimized assays.

Keywords: epididymis; mammary gland; α -lactalbumin; optimized assay; non-specific stimulation; rat

Introduction

Spermatozoa shed from the germinal epithelium in the testis are unable to fertilize eggs but achieve this property after they have traversed at least the proximal part of the post-testicular duct system (see Cooper, 1986). As they are influenced here by epididymal secretions, interference with the secretory activity of this organ may represent an approach to the development of an antifertility agent for males (Cooper *et al.*, 1986). The initial report of α -lactalbumin-like activity in the rat epididymis (Hamilton, 1981), later confirmed in rats, mice and rabbits (Jones & Brown, 1982; Byers *et al.*, 1984a, b; Limpaseni & Chulavatnatol, 1986; McLaughlin & Shur, 1987), was followed by speculation on physiological roles for the protein in regard to sperm–zona recognition (Shur & Hall, 1982; DeGeyter *et al.*, 1989) and sperm motility (Olds-Clarke, 1984; DeGeyter *et al.*, 1989). As α -lactalbumin was hitherto only thought to be present in lactating mammary glands, agents which interfere with the synthesis of this protein in the male may display antifertility effects without side-effects.

To investigate whether such an approach to male contraception is feasible, the regulation of α -lactalbumin in male tissues has to be understood and in this regard a sensitive and specific assay for α -lactalbumin was developed (Hölpert & Cooper, 1990) and used here to study α -lactalbumin-like activity in the rat epididymis.

*Reprint requests to T. G. Cooper.

Materials and Methods

Reagents. For chemicals used see Hölpert & Cooper (1990). Phenylmethylsulphonylfluoride (PMSF), iodoacetamide, *n*-octyl-glucopyranoside (nOBG), DNase I and II and collagenase were obtained from Sigma Chemie (Diesenhofen, FRG); Triton X-100 was from Pierce (Oud Beijerland, Netherlands); the glycosidase inhibitor mannonic acid gamma-lactone was from Koch Light Labs Ltd (Haverhill, UK) and Visking dialysis tubing 8/32 (M_r 10 000 cut-off) was from Serva (Heidelberg, FRG).

Animals, tissue fluids and homogenates. Adult Sprague–Dawley white rats were decapitated, trunk blood was centrifuged at $2800\text{ g}_{\text{max}}$ for 20 min at 4°C and serum was stored at -20°C . Livers, kidneys, testes, epididymides and ovaries and mammary glands from 7–10-day lactating rats were frozen and stored at -20°C . Liver, kidney, serum and gonads from adult male and female rats and mammary glands and ovaries from 7–10-day lactating rats were homogenized in Triton-free buffer (to eliminate the possibility of detergent-related activation of α -lactalbumin activity) with or without addition of authentic α -lactalbumin as internal standard. Some epididymides and testes were extracted without prior freezing.

Frozen and fresh tissues were thawed and homogenized (3 ml buffer/g tissue) in Buffer A (80 mM-Tris, pH 7.5, containing 150 mM-NaCl), Buffer B (20 mM-Tris, pH 7.4 with 10 mM-MgCl₂, 1% (v/v) Triton X-100) or Buffer C (20 mM-Hepes, pH 7.4, with 10 mM-MgCl₂, 2 mM-PMSF, 10 mM-iodoacetamide, 0.1 mg BSA/ml, 0.56 mg penicillin/ml, 0.94 mg streptomycin/ml, 10 mM-D-mannonic acid gamma-lactone and 40 mM-nOBG). Homogenization was effected on ice with an Ultra-turrax TP 18/10 (Janke & Kunkel KG, Breisgau, FRG) run for 3×10 sec with 30 sec cooling between, followed by sonication (Sonikator KLN 250/106, Ultraschall GmbH, Heppenheim, FRG) run at maximum power output for 3×15 sec with 45 sec cooling between.

Crude epididymal fluid was obtained by mincing fresh epididymides and washing tissue pieces in Buffer A (2 ml/g) for 1 h at 4°C . Homogenates and epididymal fluid were centrifuged twice at $100\,000\text{ g}$ for 1 h (4°C), supernatants were filtered through glass wool and stored at -20°C . Rete testis fluid was collected 18–24 h after ligation of the efferent ducts (Tuck *et al.*, 1970).

Ammonium sulphate precipitation. Precipitates from ammonium sulphate treatment (Cooper, 1977) were dissolved in Buffer B containing no Triton or in affinity chromatography coupling buffer (Barker *et al.*, 1972). Desalting and equilibration with affinity chromatography coupling buffer was achieved by gel filtration with Sephadex G-25 (PD-10) columns.

Galactosyltransferase affinity chromatography. Affinity chromatography utilizing *N*-acetylglucosamine hexanolamine-agarose was performed as described by Barker *et al.* (1972) with a volume of wet adsorbent of 3 ml/column (Qasba *et al.*, 1983). Non-absorbed effluents from the affinity columns (12 ml) were concentrated with Centricon-10 membrane filters.

Anion exchange chromatography. Epididymal extracts (5 ml) homogenized in Buffers A or C, or homogenized in Buffer B and subsequently precipitated by 40–60% ammonium sulphate, were dialysed twice against 2 litres start buffer (20 mM-Tris-HCl, pH 7.1). The retentate was centrifuged (30 min, $100\,000\text{ g}$, 4°C) and 2 ml supernatant were subjected to Fast Protein Liquid Chromatography on a Mono Q anion exchange column (Pharmacia). Proteins were eluted by a salt gradient (0–400 mM-NaCl) in 1-ml fractions and desalted on PD-10 columns. Freeze-dried samples were taken up in deionized water for analysis.

Thin-layer chromatography. Thin-layer chromatography was performed as described by Hölpert & Cooper (1990).

Cell culture medium. Apical and basal media from monolayer cultures of immature rat epididymal epithelium (Cooper *et al.*, 1989) were frozen at -20°C .

α -Lactalbumin assays. The incubation protocols of 3 assays were compared: the optimized assay of Hölpert & Cooper (1990) and the non-optimized assays of Hamilton (1981) and Fitzgerald *et al.* (1970a: see Table 1). In each case products were separated by addition of Dowex, as described by Hölpert & Cooper (1990). Abbreviations for the optimized assay are as follows: (i) LA_{tiss} = α -lactalbumin assay (measures transfer to glucose by sample or standard in presence of excess GTase); (ii) NS_{tiss} = non-specific reactions (measures breakdown of substrate by sample during LA_{tiss} assay), performed as the LA_{tiss} assay but with GTase omitted; (iii) LSA_{end} = inherent lactose synthetase activity of added GTase (measures transfer to glucose by GTase alone), performed as the LA_{tiss} assay but with sample omitted; (iv) Mn = manganese catalysed decomposition of substrate, performed as for NS_{tiss} but with the sample omitted.

Statistical analysis. Statistical analysis was by simple linear regression and one-way analysis of variance (least significant differences method) at a significance level of 5%.

Experiment 1. A pH profile of α -lactalbumin activity was determined with the optimized assay for mammary gland homogenates diluted 1:16 with the extraction buffer and undiluted epididymal homogenates.

Experiment 2. Attempts were made to unmask latent α -lactalbumin activity by (a) diluting out possible inhibitors (1:2 in Buffer B, $n=6$), (b) freezing and thawing once, (c) treating with collagenase (0.25 mg/ml) to remove potentially interfering macromolecules, (d) concentrating culture media by ammonium sulphate precipitation ($\times 120$ concentration, $n=3$), (e) purifying samples by ammonium sulphate precipitation (fractions: 0–20%, 20–40%, 40–60%, 60–75%, >75%) followed by affinity chromatography on NAG columns or anion exchange chromatography (40–60% ammonium sulphate).

Experiment 3. The assay conditions of Hamilton (1981) were used to construct a standard curve of α -lactalbumin with bovine GTase.

Experiment 4. Analyses were made of thin-layer chromatograms for the radioactivity present after Dowex application to assays of epididymal fluid in the presence and absence of glucose under the conditions of Hamilton (1981). With the optimized assay, metabolites produced during the α -lactalbumin assay of an epididymal homogenate prepared with Buffer B were examined.

Experiment 5. The optimized assay (Hölpert & Cooper, 1990) was compared in 3 experiments with that of Fitzgerald *et al.* (1970a). For the latter assay 4 mM-ATP was included to reduce (pyro)phosphatase activity. The tissues were extracted as below with or without the addition of authentic α -lactalbumin (35 ng) as internal recovery standard.

Crude epididymal fluid and homogenates from epididymides from which the fluid had been removed (homogenized by Ultra-turrax in Buffer B) and intact epididymides (homogenized by Ultra-turrax or by hand-held homogenizer in Buffer B) were used. Standards and tissues without exogenous standards were processed in parallel. For epididymal extracts purified by GTase affinity chromatography after 40–60% ammonium sulphate precipitation, standard α -lactalbumin was added after affinity chromatography.

Experiment 6. The activity of each extract measured under non-optimal conditions in the absence of exogenous α -lactalbumin was plotted against the corresponding percentage activation of the internal standard. Tissues known to contain α -lactalbumin (lactating mammary gland and ovaries contaminated with α -lactalbumin during their removal) were also assayed in the optimized and non-optimized assays.

Experiment 7. The GTase activity in 15 μ l rete testis fluid and corresponding amounts of bovine milk GTase (40 μ U) were incubated with different BSA concentrations (0–20 mg/ml) in the presence of glucose as acceptor and the conditions of Hamilton (1981).

Experiment 8. Crude epididymal fluid was concentrated and equilibrated against 50 mM-Hepes, pH 7.5 with Centricon-10 membrane filters. Transfer to *N*-acetylglucosamine was performed under optimized conditions (Hölpert & Cooper, 1990; but with 40 μ U rat RTF GTase and 1.5 or 15 mU bovine mammary GTase) and under the conditions of Hamilton (1981) with 40 μ U rat RTF as GTase source, and of Fitzgerald *et al.* (1970a) with 1.5 and 15 mU bovine milk GTase.

Results

Experiment 1: comparison of α -lactalbumin activity in rat mammary glands and epididymal extracts with the optimized assay

The mammary gland homogenate exhibited α -lactalbumin activity throughout the entire pH range whereas epididymal extracts contained no measurable α -lactalbumin activity (Fig. 1).

Experiment 2: attempts to increase the amount of α -lactalbumin activity measured in the optimized assay

No transfer activity in excess of that of the (pyro)phosphatase blanks was found in epididymal tissue homogenates or fluids whether they had been diluted, concentrated, frozen and thawed, treated with collagenase, or purified by GTase affinity and anion exchange chromatography when measured with the optimized assay (data not shown). Peaks of apparent α -lactalbumin activity were only detectable in certain eluate fractions from column-purified extracts with the non-optimized assay (data not shown).

Fig. 1. The pH profile of α -lactalbumin activity for (a) non-diluted rat epididymal homogenate and (b) diluted rat mammary gland homogenate under optimal assay conditions. Values are mean ($\pm$ s.e.m.) α -lactalbumin activity (LA: solid bars); the inherent lactose synthetase activity of the exogenously added bovine milk galactosyltransferase (LSA_{end} : open columns) and (pyro)phosphatase activity (hatched columns) for the pH ranges produced by Mes (5.5–6.63), Hepes (6.63–7.5), Heppso (8.0) and glycylglycine (8.5–9.0).

Experiment 3: sensitivity of a non-optimized assay

For bovine milk α -lactalbumin, only standards exceeding $2.5 \mu\text{g}$ gave values that were distinguishable from background activity (Table 1).

Experiment 4: metabolites formed

Thin-layer chromatographic separation of the metabolites present at the end of non-optimal assays with rat RTF revealed activity migrating with lactose on cellulose plates but with galactose on silica (Figs 2a, b). In the absence of glucose acceptor ((pyro)phosphatase activity of the sample) radioactivity was present as two peaks migrating with lactose and galactose on cellulose plates and ahead of galactose on silica plates (Figs 2c, d). With the optimized assay, monitoring of an α -lactalbumin assay in the presence of an epididymal homogenate prepared with Buffer B revealed that the bulk of activity migrated with lactose and galactose on both CEL MN 400 and Silica Gel₆₀ (Figs 2e, f). On silica plates the mean ($\pm$ s.e.m.) R_f for lactose was 0.17 ± 0.008 , i.e. similar to the migration found by Gauch *et al.* (1979: R_f 0.20).

Experiment 5: measurement of internal standards

Epididymal extracts prepared under a variety of conditions expressed only the inherent α -lactalbumin activity of the exogenous GTase (LSA_{end}) when assayed under optimized conditions.

Table 1. Conditions for α -lactalbumin assays used for epididymal tissue

	References*						
	1	2	3	4	5	6	7
Mn ²⁺ (mM)	9	3	3	3	3	3	10
Glucose (mM)	25	25	20	25	25	25	30
KCl (mM)	0	0	0	0	0	0	5.4
NaCl (mM)	0	0	0	0	0	0	128
ATP (mM)	4	0	1	0	0	0	0
Hepes (mM)	50	50	50	50	50	50	20
pH	6.63	7.5	7.0	7.5	7.5	7.5	7.2
UDPGal (mM)	0.4	0.4	0.4	0.4	0.4	0.4	0.1
[³ H]UDPGal (μ Ci)	0.1	—	—	—	—	—	1.0
[¹ H]UDPGal (μ Ci)	—	0.004	60.1	8.2	8.2	†	—
GT (μ U)	5800	10 ⁶	10 ⁵	30	30	2.10 ⁵	0.19
Source of GT	BM	BM	BM	RTF	RTF	BM	BM
Purified?	Partly	Partly	Partly	No	No	Partly	Yes
Sol. buffer (%)	24.5	0	0	0	0	0	0
Total vol. (μ l)	100	100	100	75	75	120	50
Sample (μ l) or wt	10	10	5	28	28	60 μ g	50 μ g
Time (min)	120	5–20	5	120	60	120	30
Temp. (°C)	35	37	37	35	35	35	37
End reaction	D	BW	BW	IE	IE	IE	E
Separation	D	CC	CC	CC	CC	CC	PE
PPase check?	Yes	No	Yes	Yes	Yes	No	No
LSA _{end} correction?	Yes	Yes	No	No	No	No	Yes
Sensitivity (ng)	2.7	3000‡	—	2500	—	—	—
Specificity check?	Yes	No	No	Yes	Yes	No	No

PPase = (pyro)phosphatase activity; BM = bovine milk; RTF = rat rete testis fluid; sol. = solubilization: 50 mM-Hepes pH 7.5, 0.1% BSA, 0.1% Triton X100, 50% glycerol; D = add Dowex; BW = boiling water; I = ice; E = EDTA; CC = column chromatography; PE = high voltage paper electrophoresis.

*1, Hölpert & Cooper (1990); 2, Fitzgerald *et al.* (1970a); 3, Byers *et al.* (1984a); 4, Hamilton (1981); 5, Jones & Brown (1982); 6, Limpaseni & Chulavatnatol (1986); 7, McLaughlin & Shur (1987).

†Amount not given.

‡ng per ml.

For each extraction the activity was the same and no treatment of the epididymal tissue revealed true α -lactalbumin activity. With the non-optimized assay (Fitzgerald *et al.*, 1970a), apparent α -lactalbumin activity was present in all tissue fractions with the exception of the samples rendered free of endogenous GTase by NAG affinity chromatography (Table 2).

When standards were added to the samples before extraction the difference between the estimates of total α -lactalbumin activity and that in tissue alone provided by the optimized assay was equal to the value of the added standard. With the unoptimized assay, on the other hand, activity of the epididymal fluid fraction, which contained no detergent, was significantly higher than the standard and even higher values were obtained from extracts containing Triton (Table 2).

Experiment 6: α -lactalbumin activity in other tissues

Under the optimized conditions α -lactalbumin activity was only expressed in lactating mammary glands and serum (Table 3). In contrast, activity was expressed in every tissue with the non-optimized assay. A plot of the activity in the tissue measured under non-optimal conditions against the extent of activation of added standards revealed striking linear relationships for tissues from males and females both with and without added α -lactalbumin (Fig. 3). The slope obtained from male rat tissues (21.84 ± 0.66 d.p.m./% activation) was greater than that for the female

Fig. 2. The location on thin-layer chromatograms from origin (left arrow) to solvent front (right arrow) of radioactive metabolites generated during assays of α -lactalbumin in epididymal tissue with rat RTF GTase. Data are from the presence (**a, b**) and absence (**c, d**) of glucose under non-optimal conditions and in the presence of glucose under optimal conditions (**e, f**). Separation on cellulose plates (**a, c, e**) of (dark bars under traces) [1] UDP-galactose, [2] galactose-1-phosphate, [3] galactose-1,2-cyclic phosphate, [4] lactose and [5] galactose or on silica plates (**b, d, f**) of (dark bars under traces) [1] lactose and [2] galactose.

(9.02 d.p.m./% activation). Values obtained from tissues to which standards were added were higher for both sexes (40.59 ± 0.67 d.p.m./% activation [males]; 27.78 ± 1.09 d.p.m./% activation [females]).

In contrast, when tissues known to contain α -lactalbumin were assayed with the non-optimized assay they did not fall on the regression lines since no relationship between α -lactalbumin content and stimulation of standards was observed (Fig. 3).

Experiment 7: apparent stimulation of activity towards glucose

The GTase activity in rete testis fluid (15 μ l) and of bovine milk GTase were stimulated weakly, but significantly, by BSA (concentrations of 2, 1 and 0.5 mg/ml with bovine GTase and of 2 and 4 mg/ml with RTF GTase) in the presence of glucose as acceptor and non-optimal conditions.

Experiment 8: transfer activity towards *N*-acetylglucosamine

With increasing amounts of epididymal fluid under non-optimal conditions, total transfer to NAG as acceptor with the RTF enzyme (40 μ U) remained constant. However, an increase in (pyro) phosphatase activity was responsible for the (59%) decrease in *N*-acetylglucosamine synthesis (Fig. 4). Under optimized conditions with the same activity of bovine GT no such changes were observed

Table 2. Comparison of apparent LA, LSA_{end} and activity of internal standards added to rat epididymal tissues under optimized and non-optimized conditions

Extract*	Activity ¹							
	Lactose synthetase ²		α -Lactalbumin ³		Internal standard ⁴		% Stimulation ⁵	
	Optimal ⁶	Other ⁷	Optimal	Other	Optimal	Other	Optimal	Other
F	9712 (336)	3690 (9)	-727 (245)	835 (28)	5677 (276)	10 945 (91)	-7.4	+40
H-F	9538 (202)	7468 (169)	258 (79)	12 820 (66)	—	—	—	—
H _U	9538 (202)	7468 (169)	-6 (70)	14 914 (66)	5203 (388)	23 374 (593)	-15.1	+198
H _H	9538 (202)	7468 (169)	-57 (86)	12 694 (39)	5467 (252)	23 608 (161)	-10.8	+201
NAG	10 933 (224)	10 685 (63)	117 (120)	-388 (139)	6383 (214)	20 172 (299)	+3.6	+157
STD					6131 (158)	7843 (97)		

¹d.p.m./200 μ l supernatant, mean (s.e.m.), $n = 3$.²Inherent lactose synthetase activity (LSA_{end}) of the added galactosyltransferase.³True α -lactalbumin activity [total activity, $LA = La_{tiss} - LSA_{end} - NS_{tiss} + Mn$].⁴Difference between α -lactalbumin value for tissue with 35 ng internal standard and tissue alone.⁵Recovery of internal standard [$((\text{tissue} + IS) - \text{tissue})/IS \times 100 (\%)$].⁶Optimized α -lactalbumin assay conditions (Hölpert & Cooper, 1990).⁷Non-optimized α -lactalbumin assay conditions (Fitzgerald *et al.*, 1970a) with 4 mM-ATP.*F = crude epididymal fluid; H-F = epididymal homogenate after removal of crude epididymal fluid, prepared by Ultraturrax; H_U = epididymal homogenate prepared by Ultraturrax; H_H = epididymal homogenate prepared by hand-held homogenizer; NAG = 40–60% ammonium sulphate precipitate of an epididymal homogenate, purified by galactosyltransferase affinity chromatography on *N*-acetylglucosamine-agarose; STD = 35 ng authentic bovine milk α -lactalbumin standard.

(Fig. 4). Similar observations were made with either 1.5 or 15 mU bovine GTase (decreases of 46% and 25%, respectively).

Discussion

In view of the many reports of α -lactalbumin-like activity in the epididymis of many species, the failure to find it in the rat epididymis in this study was surprising. This cannot reflect low assay sensitivity since this is an order of magnitude greater than those used by others (Hölpert & Cooper, 1990). Because the optimized assay had been validated with purified mammary gland α -lactalbumin, the possibility was checked that the activity of the epididymal protein differed in a major way from that of mammary origin. However, the assay was clearly able to detect α -lactalbumin in mammary gland homogenates over a range of pH values, but consistently failed to demonstrate activity in the epididymis.

Although free radioactivity was always detected in the Dowex supernatants, the difference between the total activity and the lactose synthetase activity of the exogenous GTase never exceeded the level of the (pyro)phosphatase activity of the tissue. This is an important observation since GTase is known to have inherent α -lactalbumin activity (Brew *et al.*, 1968; Fitzgerald *et al.*, 1970a, b) and correction for it in any assay must be made (Hölpert & Cooper, 1990; Fitzgerald *et al.*, 1970a). Since no internal control for the effect of the assayed tissue on the lactose synthetase activity of the endogenous GTase is possible, it is obligatory to use an assay in which no further stimulation of galactosyltransferase can be elicited. Insofar as activity is further stimutable only by the detergent *n*-octyl-glucopyranosidase, the assay used in this study fits this criterion.

Table 3. Comparison of apparent LA, LSA_{end} and activity of internal standards added to different tissues under optimized and non-optimized conditions

	Activity ¹					
	α -Lactalbumin ²		Internal standard ³		% Stimulation ⁴	
	Optimal ⁵	Other ⁶	Optimal	Other	Optimal	Other
Male liver	548 (126)	3826 (42)	2972 (234)	6692 (542)	-37.1	+256.9
Male kidney	227 (88)	2052 (62)	4040 (88)	5096 (230)	-14.5	+170.3
Male serum	1761 (136)	693 (32)	10 281 (217)	4003 (54)	+117.5	+113.5
Testis	35 (137)	1292 (30)	3554 (77)	4513 (246)	-24.8	+140.7
8-day lactating mammary gland	14 118 (527)	3649 (86)	5919 (132)	2128 (184)	+25.2	+13.5
Ovary	133 (162)	—	3469 (135)	4714 (69)	-26.5	+151.4
Female liver	226 (149)	1566 (168)	3607 (264)	5629 (251)	-23.7	+200.2
Female kidney	317 (114)	2396 (137)	3993 (291)	7817 (151)	-15.5	+316.9
Female serum	1893 (59)	261 (52)	11 701 (181)	3370 (114)	+147.6	+79.7
			4726 (296)	1875 (86)		

¹d.p.m./200 μ l supernatant, mean (s.e.m.), $n = 3$. In these experiments inherent lactose synthetase activity (LS_{end}) of the added galactosyltransferase was 9168 ± 63 for the optimal assay and 2495 ± 64 for the suboptimal assay.

²True α -lactalbumin activity [total activity, $LA = La_{tiss} - LSA_{end} - NS_{tiss} + Mn$].

³Difference between LA_{tiss} value for tissue with internal standard and tissue alone.

⁴Recovery of internal standard [(((tissue + IS) - tissue)/IS) $\times$ 100 (%)].

⁵Optimized α -lactalbumin assay conditions (Hölpert & Cooper, 1990).

⁶Non-optimized α -lactalbumin assay conditions (Fitzgerald *et al.*, 1979a) with 4 mM-ATP.

*STD = 35 ng authentic bovine milk α -lactalbumin standard.

A third possibility to be explored was that epididymal tissue somehow masks or prevents expression of true α -lactalbumin activity as measured with the new assay. However, it has been shown from the similarity in slopes of standard curves generated in the presence of epididymal extracts that epididymal tissue does not interfere with the measurement of authentic α -lactalbumin (Hölpert & Cooper, 1990). The present study confirmed that there was neither inhibition nor non-specific stimulation of authentic α -lactalbumin activity by epididymal tissues under the optimized conditions and demonstrated that there is no additional α -lactalbumin activity in epididymal tissue.

Focus was then directed to the assays used by others. Although Fitzgerald *et al.* (1970a) recognized that a detergent could stimulate GTase activity, they omitted it from routine assays of extracts and others have done the same. Evidence that the optimized and non-optimized assays measured different activities came from observations that α -lactalbumin-like activity was only detected with the non-optimized assay in certain column purified fractions of epididymal material and from the measurement of internal standards, when the activity exceeded the value derived from the pure standard. This clearly highlights a problem with non-optimized assays in that the activity

Fig. 3. The apparent α -lactalbumin activity of sera and different tissue homogenates from normal male and 8-day lactating rats measured under non-optimal conditions (Fitzgerald *et al.*, 1970a) in the presence of 4 mM-ATP. Regression lines are for tissue from males (●, ○) and females (▲, △) with (●, ▲) and without (○, △) internal standards. Data from the rat mammary gland (■, □) and the ovary (◆), do not fit the regression lines.

Fig. 4. The influence of epididymal fluid on phosphatase activity and *N*-acetylglucosamine synthesis with RTF galactosyltransferase in a non-optimized assay. Transfer rates (ordinate) from the GTase assays (●, ○), pyrophosphatase assays (▲, △) and resulting true GTase values (■, □) for a non-optimal assay (Fitzgerald *et al.*, 1970a: solid symbols) and the optimized assay (Hölpert & Cooper, 1990: open symbols).

of authentic α -lactalbumin varied with the extraction conditions and was greatly elevated by tissue extracts or detergent. That the measured α -lactalbumin activity was eliminated when GTase was removed from the tissue is consistent with the view that stimulation of endogenous GTase activity is responsible for the effect.

The linear relationship between the apparent α -lactalbumin activity of tissues and the extent of activation of exogenous standards clearly demonstrates an artefact arising from the use of a non-optimized assay. The higher slope of the regression lines in the presence of α -lactalbumin suggests, in addition, that exogenous α -lactalbumin itself can contribute to the non-specific activation. With the exception of serum, this linear relationship enables a distinction to be made between true and apparent α -lactalbumin activity of tissues. Only those samples displaying no linear relationship in the non-optimized assay displayed activity in the optimized assay.

The criticism of non-specificity applies to all assays in which merely a transfer of radioactivity towards glucose or inositol as acceptor has been taken as evidence of α -lactalbumin activity (Jones & Brown, 1982; Qasba *et al.*, 1983; Byers *et al.*, 1984a; McLaughlin & Shur, 1987). With the exception of high voltage electrophoresis, the assays measure the amount of tracer that is not bound to the ion exchange resin at the end of incubation and chromatographic separation can thus help validate that lactose is formed by the action of α -lactalbumin. In incubations from suboptimal assays, one major metabolite was found that migrated with lactose on cellulose plates but was clearly separable from it on silica.

Few studies have included all relevant controls. Byers *et al.* (1984a) found activity in all tissues tested, perhaps attesting to the non-specific nature of the assay. In other experiments, in which rete testis fluid was used as source of galactosyltransferase activity, control proteins were tested [BSA, ovalbumin and lysozyme (Jones & Brown, 1982) and epididymal Fractions I and II (Hamilton, 1981)] and found to be less active than specific epididymal proteins or epididymal Fraction III. In these cases, however, the results could equally well be interpreted as reflecting differences in the ability of the various protein fractions to stimulate the added GTase activity; a view supported by the stimulation by BSA of the GTase activity in RTF found here.

Measurements made in the absence of acceptor (Qasba *et al.*, 1983; Byers *et al.*, 1984a) provide only an index of tissue phosphatase activity; in other work (McLaughlin & Shur, 1987) only the lactose formed was estimated (Shur & Bennett, 1979) and (pyro)phosphatase blanks were obviated. Nevertheless, with both separation methods activation of GTase by tissue would be interpreted as tissue α -lactalbumin activity.

Since GTase can be activated via an interaction with a hydrophobic domain of the enzyme (Mitranic *et al.*, 1988), some hydrophobic epididymal proteins may non-specifically activate GTase. Such phenomena may explain why Hamilton (1981) found the epididymal α -lactalbumin-like activity to reside in the protein of M_r 18 500–19 000, but not in that of M_r 24 000, whereas Jones & Brown (1982) found the purified protein of M_r 23 000 to be more active than the lower molecular mass species. This issue is further confused by the finding that an epididymal protein of M_r 22 000, thought to be the same protein studied by Jones & Brown (1987), has no α -lactalbumin activity when assayed by spectrophotometric assay (Brooks, 1985).

Two reports indicate that epididymal tissue can reduce the transfer of galactose to NAG as acceptor with both testicular and bovine GTase (Hamilton, 1981; Limpaseni & Chulavatnatol, 1986). In the experiments repeated here, however, only a partial inhibition of apparent transfer to NAG was observed and this was a major consequence of phosphatase activity of the tissue. In contrast, under optimized incubation conditions, with neither enzyme source nor activity was α -lactalbumin activity detectable as a decreased transfer to NAG.

Finally, two other lines of evidence suggesting the presence of α -lactalbumin in the epididymis have to be considered. Firstly, the use of antibodies against the mammary enzyme (Qasba & Chakrabartty, 1978) or an epididymal fraction (Klinefelter & Hamilton, 1984) in immunolocalization studies have demonstrated recognized determinants at sites that differ with the antibodies used (Byers *et al.*, 1984b; Klinefelter & Hamilton, 1984, 1985; Hamilton *et al.*, 1986; Moore *et al.*, 1987). This may reflect differences in the nature of the antigen to which the antibody was raised, for another antibody to the rat epididymal protein of M_r 22 000 did not precipitate material from rat milk (Brooks, 1985). Secondly, epididymal mRNA shares sequence homology with a cDNA probe

obtained from the rat mammary gland α -lactalbumin (Qasba *et al.*, 1983), although the signal is 50–75 times less than in 5-day lactating mammary gland.

Reports of the biochemical α -lactalbumin activity in the rat epididymis may be a manifestation of artefacts arising from problems inherent in the use of unvalidated assays. The presence in the epididymis of a protein with epitopes similar to α -lactalbumin but devoid of biochemical activity cannot be ruled out.

We thank Professor E. Nieschlag for his interest in and support of this work. It was financed by the Deutsche Forschungsgemeinschaft Grant Ni 130/11-1/C.

References

- Barker, R., Olson, K.W., Shaper, J.H. & Hill, R.L. (1972) Agarose derivatives of uridine diphosphate and *N*-acetylglucosamine for the purification of a galactosyltransferase. *J. biol. Chem.* **247**, 7135–7147.
- Brew, K., Vanaman, T.C. & Hill, R.L. (1968) The role of α -lactalbumin and the A protein in lactose synthetase: a unique mechanism for the control of a biological reaction. *Proc. natn. Acad. Sci. USA* **59**, 491–497.
- Brooks, D.E. (1985) Characterization of a 22 kDa protein with widespread tissue distribution but which is uniquely present in secretions of the testis and epididymis and on the surface of spermatozoa. *Biochim. Biophys. Acta* **841**, 159–70.
- Byers, S.W., Dym, M., Hewlett, I.K. & Qasba, P.K. (1984a) Alpha-lactalbumin-like proteins in the male reproductive tract. *Ann. N.Y. Acad. Sci.* **438**, 8–17.
- Byers, S.W., Qasba, P.K. & Paulson, H.L. (1984b) Immuno cytochemical localization of α -lactalbumin in the male reproductive tract. *Biol. Reprod.* **30**, 171–177.
- Cooper, T.G. (1977) *The Tools of Biochemistry*. John Wiley & Sons, New York.
- Cooper, T.G. (1986) *The Epididymis, Sperm Maturation and Fertilisation*. Springer Verlag, Heidelberg.
- Cooper, T.G., Waites, G.M.H. & Nieschlag, E. (1986) The epididymis and male fertility: a symposium report. *Int. J. Androl.* **9**, 81–90.
- Cooper, T.G., Yeung, C.H. & Meyer, R. (1989) Immature rat epididymal epithelial cells grown in static primary monolayer culture on permeable supports. I. Vectorial secretion. *Cell Tiss. Res.* **256**, 567–572.
- DeGeyter, C.D., Cooper, T.G., DeGeyter, M. & Nieschlag, E. (1989) Effects of bovine mammary alpha-lactalbumin on hyperactivation and sperm-zona pellucida binding of mouse spermatozoa. *Gamete Res.* **24**, 415–426.
- Fitzgerald, D.K., Colvin, B., Mawal, R. & Ebner, K.E. (1970a) Enzymatic assay for galactosyltransferase activity of lactose synthetase and alpha-lactalbumin in purified and crude systems. *Analyt. Biochem.* **36**, 43–61.
- Fitzgerald, D.K., Brodbeck, U., Kiosawa, I., Colvin, B. & Ebner, K.E. (1970b) Alpha-lactalbumin and the lactose synthetase reaction. *J. biol. Chem.* **245**, 2103–2108.
- Gauch, R., Leuenberger, U. & Baumgartner, E. (1979) Quantitative determination of mono-, di- and trisaccharides by thin-layer chromatography. *J. Chromatogr.* **174**, 195–200.
- Hamilton, D.W. (1981) Evidence for α -lactalbumin-like activity in reproductive tract fluids of the male rat. *Biol. Reprod.* **25**, 385–392.
- Hamilton, D.W., Wenstrom, J.C. & Moore, A. (1986) Characterization of a sperm membrane glycoprotein. *Adv. exp. Med. Biol.* **205**, 121–129.
- Hölpert, M. & Cooper, T.G. (1990) Improved assays of α -lactalbumin and galactosyltransferase. *Analyt. Biochem.* **188**, 168–175.
- Jones, R. & Brown, C.R. (1982) Association of epididymal secretory proteins showing α -lactalbumin-like activity with the plasma membrane of rat spermatozoa. *Biochem. J.* **206**, 161–164.
- Jones, R. & Brown, C.R. (1987) Identification and characterisation of the 2D6 and Mr 23 000 antigens on the plasma membrane of rat spermatozoa. *Biochem. J.* **241**, 353–360.
- Klinefelter, G.R. & Hamilton, D.W. (1984) Organ culture of rat caput epididymal tubules in a perfusion chamber. *J. Androl.* **5**, 243–258.
- Klinefelter, G.R. & Hamilton, D.W. (1985) Synthesis and secretion of proteins by perfused caput epididymal tubules, and association of secreted proteins with spermatozoa. *Biol. Reprod.* **33**, 1017–1027.
- Limpaseni, T. & Chulavatnatol, M. (1986) A rat epididymal sialoprotein with α -lactalbumin activity. *Biochem. Int.* **13**, 41–49.
- McLaughlin, J.D. & Shur, B.D. (1987) Binding of caput epididymal mouse sperm to the zona pellucida. *Devl Biol.* **124**, 557–561.
- Mitranic, M.M., Paquet, P.R. & Moscarello, M.A. (1988) The interaction of bovine milk galactosyltransferase with lipid and α -lactalbumin. *Biochim. Biophys. Acta.* **956**, 277–284.
- Moore, A., Ensrud, K., Baker, J., Wenstrom, J. & Hamilton, D.W. (1987) Preliminary observations on a second Mr $\approx$ 24 000 membrane molecule from rat spermatozoa. *Ann. NY Acad. Sci.* **513**, 204–214.
- Olds-Clarke, P. (1984) Genetic analysis of mammalian spermatogenesis: use of the t complex in the mouse in studies of spermatogenesis and sperm function. *Ann. NY Acad. Sci.* **438**, 206–216.
- Qasba, P.K. & Chakrabarty, P.K. (1978) Purification and properties of two forms of rat α -lactalbumin. *J. biol. Chem.* **253**, 1167–1173.
- Qasba, P.K., Hewlett, I.K. & Byers, S. (1983) The presence of the milk protein, α -lactalbumin, and its mRNA in the rat epididymis. *Biochem. Biophys. Res. Commun.* **117**, 306–312.

- Shur, B.D. & Bennett, D.** (1979) A specific defect in galactosyltransferase regulation on sperm bearing mutant alleles of the T/t locus. *Devl Biol.* **71**, 243–259.
- Shur, B.D. & Hall, N.G.** (1982) A role for mouse sperm surface galactosyltransferase in sperm binding to the egg zona pellucida. *J. Cell Biol.* **95**, 574–579.
- Tuck, R.R., Setchell, B.P., Waites, G.M.H. & Young, J.A.** (1970) The composition of fluid collected by micropuncture and catheterization from seminiferous tubules and rete testis of rat. *Pflügers Arch. ges. Physiol.* **318**, 225–243.

Received 23 February 1990