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Purification and characterization of testicular transferrin secreted by rat Sertoli cells

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Sertoli cells synthesize and secrete a transferrin-like protein (testicular transferrin) [Skinner & Griswold (1980) *J. Biol. Chem.* **255**, 1923–1925]. The purpose of the present study was to purify and characterize testicular transferrin and to compare it with serum transferrin. Testicular transferrin was obtained from the medium of cultured rat Sertoli cells, whereas serum transferrin was obtained from rat serum. Both proteins were purified with the use of phenyl-Sepharose hydrophobic chromatography and transferrin immunoaffinity chromatography. The purified proteins were shown to have similar molecular masses (75000Da) and amino acid compositions. The pattern of tryptic peptides from testicular and serum transferrin were found to be essentially the same when analysed by reverse-phase high-pressure liquid chromatography. The carbohydrate composition of both transferrins was determined by several colorimetric assays and g.l.c. Testicular transferrin, isolated from cell culture medium, had increased amounts of glucose, galactose and glucosamine. Serum transferrin that was incubated with cell culture medium also had a large amount of associated glucose. The results show that testicular transferrin and serum transferrin are structurally very similar and are possibly products of the same gene expressed in two different tissues, the testis and liver. However, the amount of carbohydrate associated with these two proteins is different.

Transferrins are iron-binding proteins found in the physiological fluids and cells of vertebrates (Aisen & Litowsky, 1980). These proteins are two-sited metal-binding glycoproteins with molecular masses of 75000–80000Da (Aisen & Litowsky, 1980). Transferrins have a high affinity for Fe^{3+}, with a dissociation constant of approx. 10^{-23}M (Aisen & Leibman, 1978). Because of this high binding affinity and that of other iron-binding proteins, essentially all of the iron in physiological fluids is sequestered by transferrin or these other metal-binding proteins (e.g. ferritin).

Three major types of transferrins have been described and characterized independently. Serum transferrin produced by the liver is the major iron-binding and transport protein found in serum and most physiological fluids (Aisen & Litowsky, 1980). Lactoferrin is found in milk, tears and leucocytes (Putnam, 1975). Lactoferrin has similar iron-binding properties to serum transferrin, but does not cross-react with antibodies to serum transferrin (Aisen & Leibman, 1972). Oviduct transferrin (ovotransferrin, conalbumin) is the iron-binding protein found in avian egg white (Thibodeau *et al.*, 1978). Oviduct transferrin was found to be the same gene product as avian serum transferrin (Lee *et al.*, 1980).

We have demonstrated that cultured rat Sertoli cells secrete a transferrin-like protein termed 'testicular transferrin' (Skinner & Griswold, 1980). Sertoli cells are testicular epithelial cells that are found in the seminiferous tubules and which interact with developing germinal cells. The formation of tight junctional complexes between Sertoli cells results in the formation of a functional blood/testis barrier (Waites & Neaves, 1977).

The secreted testicular transferrin was found to have an iron-binding ability and a molecular mass similar to those of serum transferrin produced by the liver (Skinner & Griswold, 1980). In addition, antisera raised to serum transferrin recognized testicular transferrin. We determined with a

Abbreviations used: h.p.l.c., high-pressure liquid chromatography; PMSF, phenylmethanesulphonyl fluoride; IgG, immunoglobulin G; SDS, sodium dodecyl sulphate; Tos-Phe-CH_2Cl, tosylphenylalanylchloromethane ('TPCK').

radioimmunoassay that a combination of hormones (follitropin, insulin, testosterone) and vitamin A regulated the secretion of testicular transferrin by cultured Sertoli cells (Skinner & Griswold, 1982).

In the present study the purification and characterization of testicular transferrin is described and the structure of this protein is compared with that of serum transferrin.

Methods

Chemicals

Rabbit antibody to rat transferrin and rat serum transferrin were purchased from Cappel Laboratories, West Chester, PA, U.S.A. Tos-Phe-CH_2Cl-treated trypsin was obtained from Worthington Biochemical Corp. Solvents for h.p.l.c. were purchased from Burdick and Jackson Laboratories, Muskegon, MI, U.S.A. All other chemicals were obtained from Sigma Chemical Co.

Cell culture and protein collection

Sertoli cells from 20-day-old rats were isolated and cultured in serum-free medium as previously described (Wilson & Griswold, 1979; Dorrington & Fritz, 1975). Medium was collected every 48h, starting on day 4 of cell culture, for up to 2 weeks. Medium was concentrated approx. 100-fold with an ultrafiltration pressure concentrator by using an Amicon YM-10 filter. Concentrated medium was desalted on a Bio-Rad P-6 gel-filtration column. PMSF (57μM) was added to media before concentration and to desalted concentrated protein solutions.

Transferrin purification

Sertoli-cell secreted proteins were concentrated and made 1.0M with respect to $(NH_4)_2SO_4$ and 50mM with respect to Tris, pH7.5. This protein was then applied to a column (8ml) of phenyl-Sepharose previously equilibrated with 1.0M-$(NH_4)_2SO_4$/50mM-Tris, pH7.5. The transferrin was eluted from this column with a low-ionic-strength buffer (50mM-Tris, pH7.5), and the remaining bound proteins were eluted with 6M-guanidinium chloride in 50mM-Tris, pH7.5. The fractions containing transferrin were concentrated and desalted by ultrafiltration and chromatography on a P-6 gel-filtration column (Bio-Rad).

Rabbit anti-(rat transferrin) IgG was coupled to CNBr-activated Sepharose 4B as previously described (Livingston, 1974). This immunoaffinity matrix was then added to the pool of concentrated transferrin and incubated at 4°C for 20h with continuous mixing. The immunoaffinity matrix was stored, incubated, and washed in 0.5M-NaCl/50mM-Tris, pH7.5. Transferrin was eluted from the affinity matrix on a fritted-glass funnel with 3vol. of 3M-NaSCN/50mM-Tris, pH7.5, at room temperature. Eluted transferrin was concentrated on a pressure concentrator and dialysed extensively. Serum transferrin was isolated from rat serum by the same procedure as described above, except that the serum was first passed over a column (1cm × 10cm) of Affi-Gel Blue (Bio-Rad) to remove the albumin.

Radioimmunoassay

Radioimmunoassays for transferrin were performed as previously described (Skinner & Griswold, 1982). No immunological cross-reactivity was found with any of the non-transferrin proteins in concentrated Sertoli-cell spent culture medium or in rat serum.

Gel electrophoresis and isoelectric-point determination

SDS/polyacrylamide-gel electrophoresis was performed with 5–15% polyacrylamide gradient slab gels (Skinner & Griswold, 1980; Laemmli, 1970).

Amino acid composition

The amino acid composition of the purified transferrins was determined on an automatic amino acid analyser (Beckman 121MB) by the Bioanalytical Laboratory Center at Washington State University. Mercaptoethanol (0.05%) was used during hydrolysis to improve methionine and glucosamine determinations (Keutmann & Potts, 1969).

Analysis of tryptic peptides by h.p.l.c.

H.p.l.c. was used to obtain one-dimensional maps of tryptic peptides. Protein samples (100μg) were reduced for 4h at room temperature in 100μl of 10mM-Tris (pH7.5)/1% (v/v) mercaptoethanol. Samples were then freeze-dried and dissolved in 100μl of 10mM-Tris, pH7.5, and 5μg of Tos-Phe-CH_2Cl-treated trypsin was added. The digestions were generally for 9h at 37°C, with samples being mixed every 30min. Samples were then centrifuged at 10000g for 10min and analysed.

H.p.l.c. was performed on a Spectra Physics 8000 instrument with a Brownlee Aquapore RP300 reverse-phase C18 column based on silica with 30nm (300Å) pores. A Beckman 160 detector with a zinc lamp was used to monitor absorbance at 214nm.

Tryptic peptides were eluted at 30°C with a flow rate of 2ml/min. The initial solvent was 15mM-phosphoric acid and the final solvent was 15mM-phosphoric acid/30% (v/v) acetonitrile. The concentration of acetonitrile was increased linearly from 0 to 30% in 120min.

Carbohydrate analysis

The amount of hexose was determined by a modified anthrone procedure (Spiro, 1966). Anthrone solution (1.0ml; 500mg of anthrone, 10μg of thiourea, 720ml of H_2SO_4 and 280ml of water) was added to 100μl of protein sample in screw-top test tubes. The solutions were incubated 30min at 110°C, cooled, and after 20min the A_{620} measured. The assay results were linear for 2–100μg of hexose.

The sialic acid content of the transferrins was measured with a modified resorcinol procedure (Keutmann & Potts, 1969). To 500μl samples, 500μl of resorcinol solution (18mM-resorcinol/9.6M-HCl/0.25mM-$CuSO_4$) was added in screw-top test tubes. The samples were incubated 30min at 110°C and the A_{580} determined. This assay was linear for 1.5–50μg of sialic acid.

The neutral sugars fucose, mannose, galactose and glucose were determined by g.l.c. Protein samples (from 250 to 500μg) were hydrolysed in 3M-HCl for 2h at 110°C in evacuated sealed ampoules. The samples were then freeze-dried, redissolved in water and applied to an ion-exchange column (0.5ml) containing Dowex 50 resin and eluted with water. The effluent was freeze-dried and 15μl of Tri-Sil Z (Pierce) silylation reagent was added. Samples were incubated at 60°C for 2h with occasional mixing and then analysed on the g.l.c. instrument, which was a Varian 2700 with a flame-ionization detector and a glass column (1.8m × 2mm) (Alltech Associates) containing OV-1. Samples were applied at 100°C and the temperature was increased at a rate of 7°C/min to 170°C, during which time the sugars were eluted. The temperature was raised to 250°C and the inositol internal standard was eluted. Helium was used as a carrier gas at 30ml/min.

The glucosamine content was determined with an automatic amino acid analyser. Various concentrations of glucose standards were hydrolysed for 24h at 110°C and the percentage recovery of glucosamine was determined to be 83%. Calculations of the amount of *N*-acetylglucosamine in the proteins were adjusted for the use of glucosamine as standard.

Results

Testicular transferrin was obtained from Sertoli-cell spent culture medium, which was concentrated by ultrafiltration and desalted by gel filtration before hydrophobic chromatography. Testicular transferrin was eluted from the phenyl-Sepharose column with the low-ionic-strength buffer (Fig. 1). After the hydrophobic chromatography, testicular transferrin was further purified by transferrin

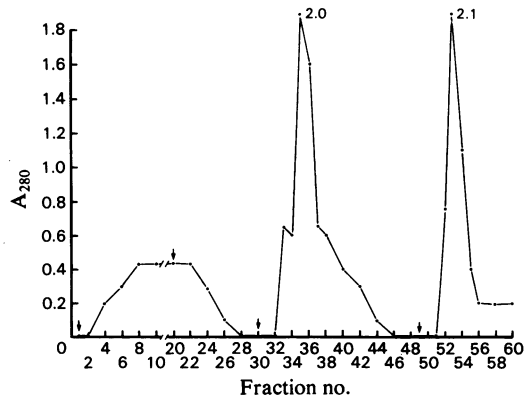

Fig. 1. *Phenyl-Sepharose hydrophobic chromatography of Sertoli-cell secreted proteins*

The column (1cm × 10cm) was equilibrated in 1.0M-$(NH_4)_2SO_4$, and a 113ml sample in 1.0M-$(NH_4)_2SO_4$ was applied. The arrows denote buffer changes at fraction 1 (sample application), fraction 20 [1.0M-$(NH_4)_2SO_4$], fraction 30 [0.0M-$(NH_4)_2SO_4$/50mM-Tris pH7.5] and fraction 49 (6M-guanidinium chloride/50mM-Tris, pH7.5). A flow rate of 1ml/min was used. Fractions (5ml each) were monitored at 280nm.

immunoaffinity chromatography. The binding capacity of 10ml of immunoabsorbent, made with 4ml of antisera, was 2mg of transferrin. The binding capacity of the affinity column was decreased by 25% each time it was eluted with NaSCN. Serum transferrin obtained from rat serum was purified by a similar procedure.

SDS/polyacrylamide-gel electrophoresis was used to analyse the proteins present at each step in the purification scheme. A single band of protein was obtained for both testicular and serum transferrin after the immunoaffinity chromatography (Fig. 2). A specific concentration (mg of transferrin/mg of protein) was calculated by comparing the total amount of protein with the amount of transferrin determined by a radioimmunoassay. The purification profiles of both serum and testicular transferrin are shown in Table 1. In general, from 10 litres of spent culture medium, approx. 1.5mg of purified testicular transferrin was obtained. The apparent molecular mass of both testicular and serum transferrin was 75000Da on the basis of SDS/polyacrylamide-gel electrophoresis.

The amino acid compositions of purified rat testicular transferrin, serum transferrin purified in this laboratory, and serum transferrin obtained from Cappel Laboratories were compared (Table 2). The amino acid composition of each of the proteins appeared to be similar. The analysis was

Table 1. *Transferrin purification profile*

Total protein was determined with a modified Lowry procedure, and total transferrin (Tf) with a radioimmunoassay (Skinner & Griswold, 1982; Hartree, 1972). Sertoli-cell secreted proteins were concentrated 100-fold (100×). Protein samples were testicular transferrin (tTf) and serum transferrin (sTf).

Sample	Volume (ml)	Total protein (mg)	Total Tf (mg)	Specific concentration (mg of Tf/mg of Protein)	Yield (%)
Secreted proteins (100×)	118	63.7	5.4	0.085	100
Phenyl-Sepharose	9.5	30.4	4.2	0.135	78
Affinity gel (tTf)	3.0	1.50	1.52	1.02	28
Rat plasma	10	336	37	0.110	100
Affi-Gel Blue	32	195	32	0.164	87
Phenyl-Sepharose	10	20.4	5.1	0.250	14
Affinity gel (sTf)	3.0	3.00	3.06	1.02	8

Table 2. *Amino acid composition of rat testicular and serum transferrin*

Protein samples were testicular transferrin (tTf), serum transferrin (sTf), and commercial serum transferrin (csTf). The values are residues per molecule to the nearest integer value, assuming 710 total residues per molecule. Values represent the mean ± 1 S.D. for four determinations on separate preparations. The data for each amino acid were analysed statistically with Duncan's (1955) multiple-range test for variable values, and no significant difference was found for each amino acid between the three protein samples at $P < 0.05$. The data were obtained from a simple time point of hydrolysis (24h) and have not been corrected for the destruction of serine or threonine.

Amino acid	Sample ...	Composition (residues/molecule) tTf	sTf	csTf
Asx		75±2	79±3	78±2
Thr		39±2	41±1	39±1
Ser		41±2	44±2	42±2
Glx		72±2	70±2	68±3
Pro		44±1	44±1	45±1
Gly		71±4	67±3	66±3
Ala		61±2	64±3	65±3
Val		47±1	46±2	47±1
Met		5±0	5±1	5±0
Ile		23±1	23±1	23±1
Leu		60±2	64±2	61±2
Tyr		22±1	23±1	21±1
Phe		32±1	35±2	33±1
Lys		56±2	58±1	58±1
His		18±1	19±1	19±1
Arg		25±1	26±1	26±1

performed on 50 μg of protein, which represented 3 nmol of methionine and 30 nmol of aspartic acid/asparagine, the least and most abundant residues respectively.

The tryptic peptides of testicular and serum transferrin were analysed and compared by reverse-phase h.p.l.c. The elution profile of the peptides obtained from a 9 h trypsin self-digest was first determined and no peaks of magnitude greater than A0.01 were found (results not shown) and it was decided that trypsin self-digestion could be ignored. The elution profiles of the tryptic peptides of testicular transferrin and of serum transferrin were then compared (Figs. 3 and 4). The peaks marked B in Figs. 3 and 4 appeared to arise from the β-mercaptoethanol which was added during the digestion procedure. The small peak marked A in Fig. 3 sometimes appeared in both testicular- and serum-transferrin peptide maps but did not consistently appear in either one. The comparison of tryptic peptide maps from testicular and serum transferrin was done six times, with no significant difference between the two peptide maps, except for the inconsistent appearance of peak A. The major peptides numbered 1–53 inclusive in Figs. 3 and 4 have essentially the same retention times, and the majority of peaks have similar magnitudes. The

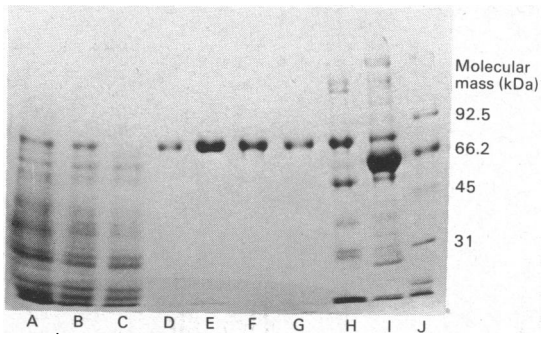

Fig. 2. *Coomassie Blue-stained 5–15%-polyacrylamide gradient gel*

The following protein samples were applied to the designated lane: A, Sertoli-cell secreted proteins; B, low-ionic-strength pool of Sertoli-cell proteins from the phenyl-Sepharose column; C, flow-through of Sertoli-cell proteins from the immunoaffinity gel; D and E, testicular transferrin eluted from the immunoaffinity gel; F and G, serum transferrin eluted from the immunoaffinity gel; H, low-ionic-strength pool of serum sample from the phenyl-Sepharose column; I, whole rat serum; J, molecular-mass standards: phosphorylase *b* (92.5kDa), bovine serum albumin (66.2kDa) and ovalbumin (45kDa).

magnitude of some of the peaks varied slightly between digests and between runs. However, the elution times, which are dependent on the amino acid composition of the peptide, did not vary.

If the transferrins were digested with trypsin for longer than 8h, the peptide maps did not change appreciably. However, there was always some undigested protein material that was eluted from the h.p.l.c. column if the acetonitrile concentration was increased to 40%.

The carbohydrate compositions of testicular and serum transferrin were found to be different (Table 3). Testicular transferrin contained 8.3% (w/w) hexose, whereas serum transferrin contained only 2.2%.

The sialic acid content of testicular transferrin and serum transferrin are the same, but testicular transferrin contains more glucosamine. In order to determine the content of neutral sugars, the hydrolysed sugars from serum transferrin and testicular transferrin were analysed by g.l.c. In addition, the neutral sugars in commercial human transferrin were analysed by the same method. The results we obtained with human transferrin (Table 3) correlate very well with the previously determined carbohydrate composition of this glycopro-

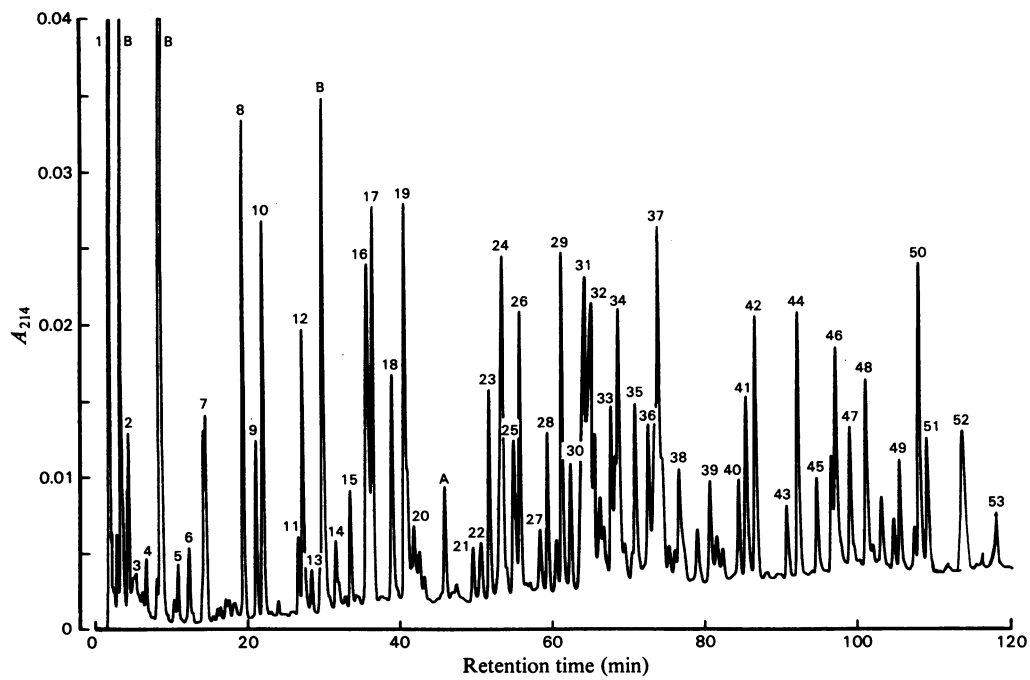

Fig. 3. *Testicular-transferrin tryptic-peptide reverse-phase h.p.l.c. profile*

Peptides were eluted with a 120min linear gradient of 0–30% acetonitrile and monitored at 214nm. Peptides were obtained after a 9h, 37°C, 5% (w/w)-Tos-Phe-CH_2Cl-treated-trypsin digestion of 100μg of protein.

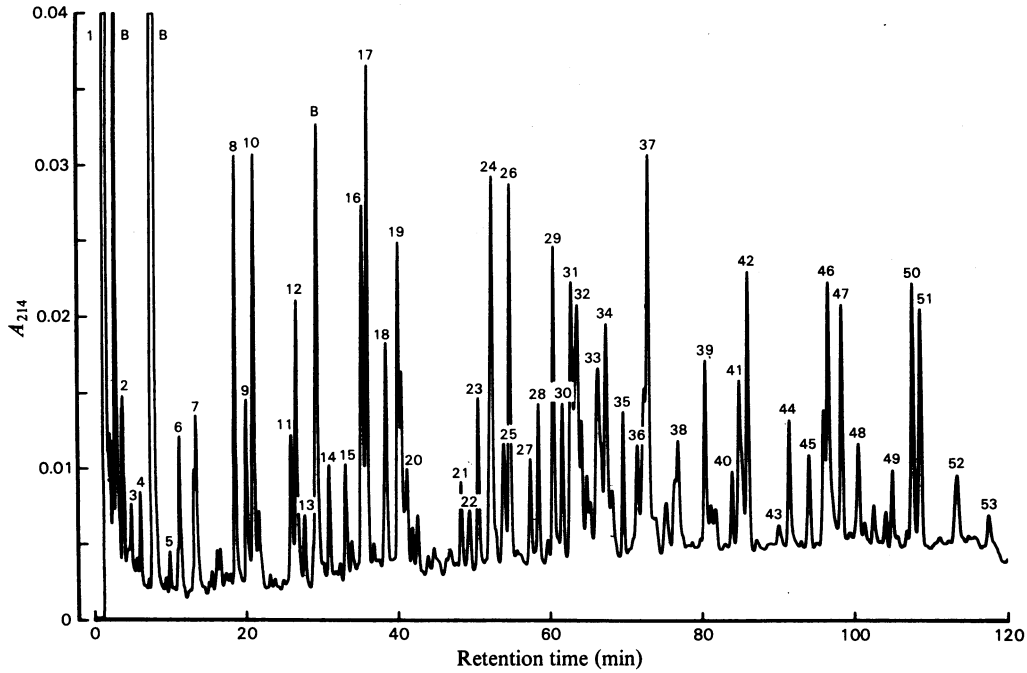

Fig. 4. *Serum-transferrin tryptic-peptide reverse-phase h.p.l.c. profile*
For details, see Fig. 3.

Table 3. *Carbohydrate composition of testicular and serum transferrin*

The method of determination is indicated by superscripts: [a]g.l.c.; [b]resorcinol colorimetric assay; [c]amino acid analyser; [d]anthrone colorimetric assay. Samples were: tTf, testicular transferrin; sTf, serum transferrin; hTf, human transferrin; and gsTf, glucosylated serum transferrin incubated in culture medium. The numbers of determinations are given in parentheses. Values are means ± 1 S.D. Abbreviation used: N.D., not determined.

		Composition (%, w/w)			
Carbohydrate	Transferrin . . .	tTf	sTf	gsTF	hTf
Fucose[a]		0.68 ± 0.05 (6)	0.7 ± 0.1 (6)	0.6 (1)	0 (3)
Mannose[a]		1.5 ± 0.1 (6)	1.5 ± 0.1 (6)	1.5 (1)	1.5 ± 0.1 (3)
Galactose[a]		2.2 ± 0.4 (6)	1.0 ± 0.2 (6)	1.0 (0)	0.9 ± 0.1 (3)
Glucose[a]		4.7 ± 0.3 (6)	0 (6)	5.4 (1)	0 (3)
Sialic acid[b]		0.9 ± 0.1 (2)	0.95 ± 0.06 (2)	N.D.	1.6 ± 0.1 (3)
Glucosamine[c]		3.2 ± 0.1 (3)	2.4 ± 0.3 (3)	N.D.	N.D.
Total hexose[d]		8.3 ± 1.5 (6)	2.2 ± 0.5 (6)	8.3 (3)	2.5 ± 0.2 (6)

tein (Spik *et al.*, 1975). The analysis of testicular transferrin and serum transferrin indicated that both contained equal amounts of fucose and mannose, but that testicular transferrin contained nearly twice as much galactose. In addition, testicular transferrin contained a large amount of glucose, a neutral sugar not commonly found in secreted glycoproteins.

The origin of this glucose was examined by incubating serum transferrin, which had no glucose in its structure, in tissue-culture medium for 2 days at 35°C. When this serum transferrin was re-isolated and analysed, it was found that the associated hexose had increased to 8.3% and that it had a substantial amount of glucose associated with it (Table 3). The glycosylated serum transferrin was incubated for 30 min in 6 M-guanidinium chloride and re-isolated by passage over a P-6 (Bio-Rad) gel-filtration column. Analysis of the sample showed that the treatment did not remove any of the associated glucose (results not shown). In addition, if serum transferrin was incubated in

[^{3}H]glucose (1 mM, 45 Ci/mmol) for 2 days at 35°C, a substantial amount of the radioactive glucose became associated with the transferrin and was not removed by a similar treatment with by 6M-guanidinium and re-isolation by gel filtration (results not shown).

Discussion

The purification of testicular transferrin was complicated by the presence of other Sertoli-cell secreted proteins which have been described (Kissinger *et al.*, 1982). Some of these proteins bind to polysaccharides and cause aggregation when the Sertoli-cell secreted proteins are concentrated. Thus gel-filtration or ion-exchange chromatography could not be used to purify testicular transferrin. However, transferrin was separated from most other secreted proteins by hydrophobic chromatography. Transferrin was eluted from the phenyl-Sepharose column with a low-ionic-strength buffer, whereas the elution of many of the other proteins required 6M-guanidinium chloride. The criteria used to determine the purity of the transferrin were a single protein band after SDS/polyacrylamide-gel electrophoresis and the specific transferrin concentration (mg/mg of protein). By both of these criteria the purification procedure yielded pure testicular and serum transferrin.

The amino acid composition of the two proteins is similar and the molecular mass appears to be the same. Tryptic peptide maps of testicular and serum transferrin as examined with reverse-phase h.p.l.c. were the same for the two proteins. Therefore the polypeptide portion of testicular and serum transferrin are probably the same.

The hexose content of testicular transferrin was higher than that of serum transferrin. Much of this difference in hexose was due to the unanticipated presence of glucose in the testicular transferrin. Since both testicular and serum transferrin were isolated by the same column procedures, it is unlikely that the glucose was a contaminant from the column matrices. When serum transferrin was incubated in culture medium in the absence of cells, it also became associated with an increased quantity of glucose. Therefore the glucose present in testicular transferrin appears to be a result of the cell-culture conditions and may not be present on transferrin *in vivo*. The nature of the interaction between glucose and the transferrins is unknown, but it may occur as a result of the interaction of glucose with the ε-amino group of lysine and the *N*-terminal amino group (McDonald *et al.*, 1978).

The carbohydrate analysis of testicular and serum transferrin showed that the quantities of fucose, mannose and sialic acid are the same in the two proteins. However, testicular transferrin contained more than twice the amount of galactose and 20% more glucosamine than serum transferrin. Thus the glycosylation of the proteins is different in the two tissues. The data we obtained for rat serum transferrin are consistent with the presence of two oligosaccharide chains with a structure similar to that reported for human serum transferrin (Spik *et al.*, 1975). In addition, the rat serum transferrin contains fucose. The data for testicular transferrin (excluding glucose) are consistent with the presence of two triantennary oligosaccharide structures similar to a glycopeptide from calf thymocyte membrane reported by Kornfeld (1978). However, the elucidation of the oligosaccharide structures must await further analysis. These results compare with those reported for chicken serum transferrin and oviduct transferrin (conalbumin, ovotransferrin), which were found to be the same gene product but differed in carbohydrate content (Williams, 1962, 1968). The functional significance of tissue-specific glycosylation is not clear.

Serum transferrin is effectively excluded from interaction with advanced germinal cells by the tight inter-Sertoli-cell junctions. Because of its low solubility in aqueous environment at neutral pH, all Fe^{3+} in living systems requires a transport vehicle such as transferrin. We have proposed that Sertoli cells synthesize and secrete testicular transferrin into the lumen of the seminiferous tubules in order to provide a source of Fe^{3+} to the developing germinal epithelium (Skinner & Griswold, 1980).

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