The Carbohydrate Structure of Porcine Uteroferrin and the Role of the High Mannose Chains in Promoting Uptake by the Reticuloendothelial Cells of the Fetal Liver*

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Philippa T. K. Saunders*, Randall H. Renegar**, Thomas J. Raub†, George A. Baumbach‡, Paul H. Atkinson*, Fuller W. Bazer§, and R. Michael Roberts¶

From the Department of Biochemistry and Molecular Biology, and §Department of Animal Science, University of Florida, Gainesville, Florida 32610 and the Department of Developmental Biology and Cancer, Albert Einstein College of Medicine, Bronx, New York 10461

Uteroferrin, the iron-containing, progesterone-induced phosphatase of the porcine uterus, is a glycoprotein carrying a single oligosaccharide chain. Most of the uteroferrin isolated from either uterine secretions or allantoic fluid has endoglycosidase H-sensitive carbohydrate chains with either five or six mannose residues. As determined by 'H-NMR spectroscopy, the Manα oligosaccharide has the following structure.

\[
\text{Man} \xrightarrow{\alpha 1,2} \text{Man} \xrightarrow{\alpha 1,3} \text{Man} \xrightarrow{\beta 1,4} \text{GlcNAc} \xrightarrow{\beta 1,4} \text{GlcNAc}
\]

The Manα species lacks the terminal α1,2-linked residue. Uteroferrin is transported across the pig placenta and has been proposed to be involved in iron transfer to the fetus (see Buhl, W. C., Ducsay, C. A., Bazer, F. W., and Roberts, R. M. (1982) J. Biol. Chem. 257, 1712–1721). Injection of 125I-labeled uteroferrin into the umbilical vein of midpregnant fetuses resulted in incorporation of label into the liver, the major site of fetal erythropoiesis. Light and electron microscope autoradiography revealed that the primary sites of uteroferrin uptake were the reticuloendothelial cells lining the liver sinusoids. Reticuloendothelial cells isolated from either fetal pig or adult rat livers were shown to accumulate uteroferrin when cultured in vitro. Uptake was inhibited by yeast mannan and by glycopeptides isolated from either ovalbumin or uteroferrin. Rat cells did not accumulate uteroferrin whose high mannose chains had been removed using endoglycosidase H. Moreover, the K uptake values (3 × 10⁻¹⁵ m), specific competition by D-mannose and L-fucose bovine serum albumin, and inhibition by EDTA are consistent with an uptake mechanism involving a receptor for high-mannose oligosaccharides on the liver sinusoidal cells. It is suggested that one function of this receptor in the fetal pig is to remove maternally derived uterine glycoproteins from the fetal circulation. In the case of uteroferrin this process provides iron to the fetal liver.

Uteroferrin (Uf) is a purple-colored glycoprotein with acid phosphatase activity (1, 2) which is synthesised by the glandular endometrium of pigs (3). Its production is under the control of progesterone (4) and it is a major secretory product of the midpregnant uterus (1). The purple coloration of Uf results from an iron center in which the metal is coordinated to one or more tyrosine residues (5, 6). There is some controversy about the iron content of different preparations of Uf, but up to two atoms can be bound (see Ref. 7). A considerable body of evidence has accumulated to suggest that a major function of Uf is the transplacental transport of iron during pregnancy (1, 3, 8, 9). In the pig, placentalization is of the diffuse epitheliocorial type (10) with several cell layers separating maternal and fetal blood supplies, an arrangement which is believed to require an indirect transfer of iron from mother to conceptus (9, 11). Special placental (chorionic) structures, known as areolae, develop opposite the mouths of uterine glands and appear to be involved in the uptake of secreted proteins (12). Uf is the major iron-containing component of porcine uterine secretions (1) and can be detected in areolae as well as in the placental venous drainage (3). The known major sites of Uf metabolism in the conceptus are allantoic fluid (8, 13) and liver (8, 9). The Uf present in allantoic fluid is thought to represent excess protein not immediately cleared from the fetal blood by the liver (3). Immunocytochemical studies have suggested that Uf forms part of the urinary filtrate and enters allantoic fluid via the bladder and urachus (3). Once there it is rapidly broken down and loses its iron to fetal transferrin (8). The liver, however, is the major site of
photocopies are also included in the microfilm edition of the Journal (14). Because many glycoproteins introduced into the blood stream of mammals are cleared by the liver by a mechanism that involves surface receptors with lectin-like specificities (15), we have examined whether the clearance of Uf from fetal blood might involve its carbohydrate. An initial analysis of Uf indicated that the majority of the molecules lacked sialic acid, but contained glucosamine, mannose, and some galactose (4). However, the presence of glucose was also reported, suggesting that some of the preparations were contaminated with exogenous carbohydrate. In this study we have re-evaluated the oligosaccharide structure of Uf and examined the role of carbohydrate in mediating the binding and retention of this glycoprotein in the liver.

MATERIALS AND METHODS AND RESULTS

DISCUSSION

The results in this paper demonstrate that the purple protein, Uf, isolated from either uterine secretions or from allantoic fluid, is a glycoprotein containing about 4.8% by weight carbohydrate. This carbohydrate is present on single oligosaccharide chains and consists mainly of high mannose structures which could be released by Endo H and which bound strongly to ConA-Sepharose. HPLC analysis revealed that the major oligosaccharides released by Endo H had the empirical formulae Man5GlcNAc and Man7GlcNAc. The structures of these oligosaccharides were determined by means of 1H-NMR analysis and are shown in Table II. Results obtained using α1,2-mannosidase for the presence of α1,2-linked, terminal mannose residues on the purified Manα and Manα oligosaccharides were entirely consistent with the assignments in Table II, i.e., a single residue of mannose was released from the Manα species but not from the Manα.

A small proportion of Uf carbohydrate appeared to be in the form of complex or hybrid-type chains as evidenced by the presence of trace amounts of galactose and sialic acid (Table I) and by the fact that a small fraction bound either weakly or failed to bind to ConA. In addition, about 5% bound to wheat germ agglutinin-Sepharose. Uf can also be labeled by the galactose oxidase-NaB₃H₄ technique, a procedure which depends upon the availability of galactosyl residues in the carbohydrate (16).

Only a very small proportion of the Uf molecules purified from allantoic fluid carried phosphorylated oligosaccharide chains. This phosphate was presumed to exist as mannose 6-phosphate since it is in this form that it is found on Uf released from cultures of uterine endometrium (17). A high proportion (up to 30%) of newly synthesized Uf carries this group, although it is masked by a covering N-acetylglucosamine residue (17). The oligosaccharide chains of newly synthesized Uf are also larger than those of the mature Uf studied here (17). We have suggested that the oligosaccharide chains on Uf continue to be modified following their release into the uterine lumen so that they progressively lose phosphate and outer saccharide residues and thus become smaller in size (17).

Extensive metabolism of Uf iron occurs in the liver of the midpregnant fetal pig (8, 9). As demonstrated here, 125I-labeled Uf introduced into the umbilical vein is taken up almost exclusively by the cells lining the liver sinusoids and not by parenchymal cells (Fig. 6). This population consists of the Kupffer and endothelial cells. In our study we have not been able to determine whether one or both of these cell types were involved in Uf uptake, and we have classified them together as reticuloendothelial cells. Uptake of 125I-labeled Uf, both in vivo and in vitro, was blocked by addition of unlabeled Uf. Although a statistical analysis was not carried out, autoradiographic studies (Fig. 5B) strongly suggested that the labeled Uf became internalized rapidly into endocytic vacuoles. Together, these experiments indicated that Uf was taken up by reticuloendothelial cells by a receptor-mediated process.

Within the past 10 years a considerable body of information has accumulated concerning the uptake of glycoproteins by mammalian cells. Of particular relevance to this study is the presence in the liver of distinct populations of receptors that bind specifically to particular carbohydrate groups (for review see Ref. 15). A receptor which binds certain oligosaccharides terminating in D-mannose, L-fucose, or N-acetylgalactosamine is found on reticuloendothelial cells of the liver (18-21) and on macrophages (22-24). Binding is characteristically inhibited by yeast mannann and by glycoproteins terminating in α-mannosyl groups. Since binding appears to require calcium, EDTA is also a potent inhibitor. Our results, using partially purified populations of reticuloendothelial cells from adult rat and fetal pig livers, suggest that the uptake of Uf is mediated by a mannos-specific cell surface receptor. For example, Uf binding to rat cells was inhibited by EDTA, by high mannose glycoproteins, and glycopeptides (Table V and Fig. 7) and by bovine serum albumin substituted with D-mannose or L-fucose residues but not with D-galactose (Table V). The kinetic constant for uptake of Uf (approximately 3 × 10⁻⁷ M) (Fig. 8) was very close to that noted for uptake of oligosaccharides terminating in mannose by rat reticuloendothelial cells (19). Although fewer experiments were carried out with equivalent cells from the fetal pig liver (Table VI), the characteristics of Uf uptake appeared to be identical to those seen with the rat cells. Therefore, even though a detailed study of the characteristics of the uptake process was not carried out, the results are entirely consistent with the hypothesis that the high mannose receptor, previously described on macrophage (22-24) and reticuloendothelial cells (18-21), mediates uteroferrin uptake by the fetal pig liver.

Binding of Uf to crude membrane fractions from fetal pig livers has also been demonstrated (3). Results from more recent experiments are consistent with the view that such binding occurs to a receptor that recognizes high mannose oligosaccharide chains and has a Kₘ of about 2.8 × 10⁻⁶ M. However, we have not pursued these studies in detail since it was impossible in our studies to determine the origin of the receptors, i.e., the cell type involved, and whether the receptors were located at the cell surface or on internal membrane systems (see Refs. 15 and 23). Their relevance in Uf uptake was therefore unclear.

The fate of Uf, once it has been taken up by the liver cells, is not known. The protein has long been believed to play partial and possibly a major role in supplying iron to the fetus until at least day 75 or so of pregnancy (1, 8, 9, 25). It is certainly synthesized during this period in amounts adequate for the requirements of fetal hematopoiesis, and its iron is readily incorporated into fetal hemoglobin (8), most of which
is synthesized in the liver. How Uf iron reaches the developing blood cells within the blood islands is unclear. Uf uptake of Fe by the reticuloendothelial cells appeared to involve coated pits and presumably resulted in internalization into an endosome compartment which was likely to have an acid pH (see Refs. 26 and 27). It had probably not entered lysosomes because of the short interval from infusion of 55Fe-labeled Uf and tissue fixation, i.e. 3–4 min. This time interval is probably insufficient for 55Fe-Uf taken up by endocytosis to be transported to lysosomes. For example, α2-macroglobulin was not detected in lysosomes until 15–30 min after exposure of fibroblast(s) to the ligand (28). A 15-min time interval was also required between exposure of rat hepatic sinusoidal cells to 55Fe-glycoproteins with terminal mannose and N-acetylglucosamine residues and the detection of these proteins in lysosomes (29).

Unlike transferrin (30–33), Uf binds its iron tightly down to pH 3 (8) and would not be expected to release its iron as a result of the low pH within the endosome or lysosome. However, while the transferrin receptor continues to bind apo-transferrin at around pH 5 (30, 31), the mannose receptor is believed to release its ligand (see Refs. 15 and 23). Thus Uf internalized on the mannose receptor would not be returned to the cell surface, as is transferrin (30–33). Rather, it would most probably move into lysosomes. It is possibly at this location that Uf is degraded and its iron is released. Alternatively, Uf may be transferred in intact form from the reticuloendothelial cells to neighboring blood islands.

In conclusion, these experiments strongly suggest that the high mannose oligosaccharides of Uf function in targeting the molecule to the fetal liver where its iron is used for erythropoiesis. Whether its carbohydrate also plays a role in uptake of Uf by the placenta, and movement of the glycoprotein in intact form across the chorionic epithelium into the placental blood capillaries, remains to be determined.

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The carbohydrate structure of Porcine Uteroferrin and the Role of the High Mannose Chains in Promoting Uptake by the Hepatocellular Carcinoma Cells of the Fetal Liver.

By P.T.K. Saunders, R.H. Benzer, T.J. Bass, C.A. Sambrook, P.H. Atkinson, F.W. Baker and R.M. Roberts

Materials and Methods

Materials. Ureagels (grade V), masses (from Baker, Las.) bovine serum albumin, lyophilized porcine Uteroferrin (Pf & from A. Allentuck, and collagenase from Streptomyces, very low protein content, Sigma Chemical Co. were obtained from Sigma Chemical Co. Endo-β-1,3-glucanase (M grade) from E. testaceus B2A and from 60-branched anhydroglucosyltransferase from B. subtilis (this protein is a limiting substrate for this enzyme) were obtained from New England Nuclear (200 units/mg). Protease inhibitors (Pefabloc SF and anti-fao) were from Pefabloc, and Bio-gel P 20 (10 x 1.5 cm) in 0.1 M acetic acid. Both buffers coorained 0.021 (w/v) sodium azide; elution of peptide fragments was followed by measuring absorbance at 280 m. Glycopeptide8 were detected by the phanol-aulfuric acid assay (42). Glycopeptide-containing fractions were pooled, dialyzed against distilled water in tubing with a molecular weight cut-off of 1000 (Spectrapor, Spectrum Medical Indw.).

To obtain oligosaccharides, glycopeptide samples (100-500 mg per 0.5 ml sugar) were dissolved in 0.5 ml of 100 mM citrate-phosphate buffer, pH 5.6, different classes of oligosaccharides were separated on a column of Mono-S Sepharose (1 x 3 x 6 cm) equilibrated with 25 mM Tris-acetate, pH 7.4, 0.15 M NaCl, 1 mM EDTA, and 0.05 M sodium azide. Elution of peptide fragments was followed by measuring absorbance at 280 nm. Glucose content was determined by the phenol-aulfuric acid assay (42). Glycopeptide-containing fractions were pooled, dialyzed against distilled water (250-500 mg Biogel P 400 (less than 400 mesh, 10 x 1.5 cm)) run in 0.1 M acetic acid, 0.021 (w/v) sodium azide; 2 ml fractions were collected.

For isolation of oligosaccharides from Uteroferrin, which had been purified by antibody affinity chromatography, and for determination of the neutral sugar content, an aliquot (0.1 ml) corresponding to 5 pg of Uteroferrin was added to a 95 ml volume of 1 M KCl buffer, pH 7.5, 0.15 M NaCl, 0.005 M sodium azide. Following application of the sample, unbound material was eluted from the gel by washing with the application buffer. Bound protein or glycopeptide was eluted by washing with buffer containing 100 mM 3'-deoxy-methylglucoside (see 31). Alternatively, 0.2 M sodium azide can be substituted for buffer containing 3'-deoxy-methylglucoside.

Analysis of carbohydrates on UF. Samples of UF were precipitated by means of 3% (w/v) trichloroacetic acid. The precipitate was washed several times with ethanol (80%) and dried at 100°C. Shredded small pieces of dried material were transferred to columns of Porcine Uteroferrin Carbohydrate.

Supplemental Material
Incorporation of reticulocytes cells. Cells were first in buffer on ice until 5 min before the start of the incubation period. Thereafter, cells for control (6°C) experiments were rewarmed to 37°C 5 min before the start of uptake experiments and transferred to a 37°C water bath. Each mL of the incubation mixture contained 0.2-1 ml labeled UF (specific activity 1-3 x 10⁶ cpm/mg) plus known amounts of competing heptan or chlorophyll. Control (tube) conditions were the same as experimental tubes; all tubes were pre-warmed to 37°C unless otherwise stated. At the start of the incubation an aliquot (7-5 x 10⁶ cells) of the cell suspension was added to each tube; final incubation volume was 1.5 or 2.0 ml. The suspensions were agitated at 5 min intervals during the 60-40 min incubation period. At the end of the incubation period, the contents of each tube were mixed and aliquots (3 x 2.5 ml) transferred to centrifuge tubes containing 1 ml ice-cold buffer. The cells were centrifuged for 2 min at 2000g in a refrigerated centrifuge. The supernatant fluid and cell-associated radioactivity determined using a gamma counter.

For determination of uptake of UF, unpaired 4 x 10⁶ cells, 2 x 10⁶ cpm [14C]-labeled UF and 0.04-0.6 μl unlabeled UF purified by Con A-Sepharose chromatography; final incubation volume 0.5 ml. Incubations were performed in triplicate. After 15 min incubation at 37°C, cell cold buffer (1 ml) was added to each tube; cells were centrifuged and radioactivity the cell pellet determined as described above.

RESULTS.

Carbohydrate composition of UF. The monosaccharide composition of UF was determined following methylation and trimethylsilyl derivatization (Table 1). The major sugar identified were N-acetylglucosamine and mannose, with traces of galactose, fucose and stachyose also present. The analysis was consistent with the view that UF molecules carry a single chain of approximate composition (Man)₃(α1,6)GlcNAc, comprising about 4.5% of the dry weight of the protein. The carbohydrate composition of UF purified from uterine secretions appeared identical to that prepared from allantoic fluid of Day 60 conceptuses.

Table 1. Monosaccharide composition of uteroferrin purified from uterine secretions and allantoic fluid.

| Monosaccharide | 1st | 2nd | 3rd |
|----------------|-----|-----|-----|
| Fucose         | Trace| Trace| 0.6 |
| Mannose        | 6.4 | 7.5 | 6.4 |
| Galactose      | 0.8 | Trace| 0.8 |
| N-acetylglucosamine | 1.7 | Trace| 1.7 |
| N-acetylmuramic acid | Trace| Trace| 0.2 |
| Percent carbohydrate | 4.12 | 4.38 | 5.01 |

*Sample purified from allantoic fluid; **different samples of uteroferrin purified from uterine secretions.

Binding of UF to lectin affinity columns. A large proportion of purified UF bound to Con A Sepharose (Fig. 3). A small fraction (0.63% of the total phosphate activity and 1.1% of the protein) failed to bind. Elution with 0.01 M D-mannitol phosphate at room temperature released 1.1% of the acid phosphatase and 15.9% of the protein. This fraction presumably contained proteins with complex chains (43,56). The remainder bound avidly and could only be removed with 0.1 M (D-mannitol) mannose if elevated temperatures (50-60°C) were employed. Elution may also be performed with 0.2 M acetic acid or brief acid treatment does not denature either the Con A or the UF, and the latter retains its purple color and full enzymatic activity.

![Figure 1](image1.png)

**Figure 1.** Chromatography of UF on a column of Con A Sepharose. UF (1.5 mg) was loaded onto a column (1 x 5 cm) of Con A Sepharose and the column was washed with buffer (2 mg Tris-HCl pH 7.6, 0.1 M NaCl, 0.1 mg D-mannitol). Bound enzyme was eluted first with 0.01 M D-mannitol phosphate and 0.01 M D-methylmannoside (G) followed by 0.01 M D-glucosylmannoside (H). The latter elution was carried out at 4°C, 3.5 min from the first elution. Protein was detected by monitoring absorbance at 280 nm (A); acid phosphatase activity (M) was assayed by removing 0.1 ml aliquots from each fraction using the method of Schlossman et al. (22).

The UF which bound tightly to Con A probably consisted of molecules carrying either high mannose or complex chains. Since leaves of the medium column to wheat germ agglutinin (immobilized on Sepharose (results not shown) was highly purified but the allantoin UF is known to be of the high mannose type. Number of oligosaccharide chains. To confirm that UF possesses a carbohydrate chain sensitive to endo H, a sample of the glycoprotein was radiolabeled and allowed to bind to Con A Sepharose. The labeled material was eluted under conditions appropriate for the recovery of UF with high mannose chains (see above) and incubated with endo H for 24 h. At various time intervals samples were removed and analyzed by DIX-FACE and autoradiography (Fig. 2).

![Figure 2](image2.png)

Three main regions of radioactivity were detected. The first appeared at a point corresponding to the position of the Man₃GlcNAc₂ standard. This was closely followed by a smaller radioactive peak (M, GlcNAc) which contained the highest amount of the radioactivity. The third peak was tentatively assigned to the Man₃GlcNAc₂ material. When larger amounts of endo H were employed, the same peaks could be demonstrated using the phenol-sulfuric acid method for detecting carbohydrates (results not shown). The integrity of each of the radioactive peaks shown in Fig. 2 was confirmed by high performance liquid chromatography. Fractions within each peak were collected, concentrated and rechromatographed on Bio-Gel P-2 columns (Fig. 3). The fractions eluted at positions identical to those of Man₃GlcNAc₂, Man₃GlcNAc and Man₃GlcNAc standards derived from IPM (43).

![Figure 3](image3.png)

Data for gel filtration of UF (4, 5). Reduced, Con A positive, oligosaccharides derived from UF on a column of Bio-Gel P-2. Oligosaccharides were isolated from UF by sequential digestion with Pronase and endo H, labeled with sodium H₂[¹⁴]O, and derivatized on a column of Sephadex G-25 to remove salts and proteins, loaded onto Con A Sepharose, and high mannose chains eluted using acetic acid. At the completion of the acetic acid wash, the glycoprotein was treated with endo H to release oligosaccharides. The latter were then reduced with NaB/H, and rechromatographed on a column of Bio-Gel P-2 (Fig. 3).

Although the experiment failed to demonstrate unequivocally that there were no intermediate forms generated between the fully glycosylated starting material (Mr 35,300) and the glycoform (Mr 35,000), the carbohydrate was clearly sensitive to endo H cleavage at reducing terminal mannose.

Analysis of endo H sensitive oligosaccharides. Glycopeptides released from UF by Pronase digestion were chromatographed on a column of Sephadex G-25 to remove salts and proteins, then loaded onto Con A Sepharose, and high mannose chains eluted using acetic acid. After removal of the acetic acid wash, the glycopeptides were treated with endo H to release oligosaccharides. The latter were then reduced with NaB/H and rechromatographed on a column of Bio-Gel P-2 (Fig. 3).
Figure 4. Analysis of 87S-labeled oligosaccharides by high performance liquid chromatography (HPLC) on a column of TSK-SW 3000 before and after treatment with 9,1,2-mannosidase. Con A-Sepharose 6B-labeled oligosaccharides were purified by gel filtration chromatography on Bio-Gel P-4 (see Fig. 4) and the positive Man, Gal, and Man, Gal, GlcNAc di- and tetra-antennary species are shown before and after enzyme treatment.

Table 2. Chemical shifts of anomeric, C-2 and $\alpha$-acetyl protons in uteroferrin oligosaccharides

| Residue Number | C-2 H | $\alpha$-M | C-2 H |
|----------------|-------|-----------|-------|
| 1              | 4.118 | 4.057     | 2.042 |
| 2              | 4.118 | 4.057     | 2.042 |
| 3              | 4.118 | 4.057     | 2.042 |
| 4              | 4.118 | 4.057     | 2.042 |
| 5              | 4.118 | 4.057     | 2.042 |
| 6              | 4.118 | 4.057     | 2.042 |
| 7              | 4.118 | 4.057     | 2.042 |
| 8              | 4.118 | 4.057     | 2.042 |

Figure 5. $^1$H-NMR spectrum of the positive Man, (a) and Man, Gal, GlcNAc oligosaccharides isolated from uteroferrin. Assignments of monosaccharide residues to numbered peaks are presented in Table 2.

Light microscopy autoradiography. Three min after injection of $^{[3]H}$-labeled UF into the umbilical vein, fetal liver was excised, fixed in 4% formalin, and sectioned (2). Autoradiographs prepared for light microscopy revealed a heavy accumulation of silver grains present over cells lining the liver sinuoids, (i.e. Kupffer and endothelial cells, see Fig. 6A). By contrast, the density of grains over the larger pericentral cells of the liver was about the same as over areas not represented by tissue, i.e. background level (Fig. 6B). Similarly, regions containing erythrocytic tissue were not heavily labeled. Worn sections of heart tissue (not shown) from the same fetuses were examined and were found not to have accumulated any $^{[3]H}$-labeled UF as detected by autoradiography. Injection of 5 mg of unlabeled UF into the umbilical vein 1 min prior to injection of $^{[3]H]$-labeled UF reduced the concentration of silver grains over sinusoidal cells to background levels (results not shown).

Electron microscopy autoradiography. The distribution of $^{[3]H}$-labeled material within the fetal liver follows injection of $^{[3]H}$-labeled UF was also examined with electron microscopy autoradiography (Fig. 6B). An analysis of the relative distribution of silver grains over different cell types was made on a series of sections and results were presented in Table 3.

Table 3. Distribution of silver grains over sections of pig fetal liver

| Cell Type               | Cell Area | Number of Silver Grains | Distribution of Silver Grains per Unit Area |
|-------------------------|-----------|-------------------------|--------------------------------------------|
| Hepatocellular (86)     | 645        | 6.5                     | 0.2500-0.39                                |
| Parenchymal (302)       | 35         | 14.6                    | 0.0200-0.001                               |
| Erythrocyte             | 60         | 0                       | 0.6                                        |
| Pro-erythrocyte (25)    | 0         | 0                       | 0.6                                        |

The results were obtained from micrographs at a constant magnification (x 4450). The number of silver grains per unit cell area were determined using a high plexiglass (Hewlett Packard) interfaced with a Model 7100A, Microcomputer. Silver grains located on junctions between cells were not counted.

It is clear that some 85% of the $^{[3]H}$-labeled UF in the liver had become associated with the reticulendothelial cells lining the sinusoids, while little radioactivity was incorporated into the parenchymal cells or intravascular erythrocytes found in the blood islands (Fig. 6B). However, on the basis of their morphology alone it was not possible to distinguish between the endothelial and Kupffer cells.

Although a detailed statistical distribution was made, we observed that the site of the radiolabeled material associated with the plasma membrane of the reticulendothelial cells. Sites of labeled protein into the cell appeared to have occurred within the 5 min before fixation, and silver grains were associated with both coated and uncoated vesicles as well as areas of coated membranes on the cell surface (insert on Fig. 6B).

Samples of $^{[3]H}$-labeled UF with reticulendothelial cells isolated from adult rat liver, because of their rapid availability and proven effectiveness for their isolation, reticulendothelial cells from rats were used in most of the experiments. The results of a typical experiment in which $^{[3]H}$-labeled UF was incubated with rat reticulendothelial cells are shown in Fig. 7.
In incubations carried out at 37°C, cell-associated radioactivity continued to rise throughout the incubation period. At 15 min, 9.4% of the total 125I provided had become bound to the cells; at 60 min, this value had increased to 19.6%. If cells were retained on ice the amount bound at 15 min was only about 3.6% of the total, and there was no increase beyond 30 min. Addition of unlabeled UF depressed uptake by about 65% by the end of 1 h. Yeast mann and ovalbumin also inhibited the accumulation of [125I]-labeled UF by the cells.

## Table 4. Comparison of the uptake of intact, glycosylated UF with UF treated with endo H.

| Protein added to cells | Temperature (°C) | Percent uptake of [125I]-labeled-aglyco-UF |
|------------------------|-----------------|------------------------------------------|
| 125I-UF                | 37              | None                                     |
| 37                     | 24 µM UF        | 15.6 ± 0.06                              |
| 4                      | None            | 7.0 ± 0.10                               |
| 125I-aglyco-UF         | 37              | None                                     |
| 37                     | 24 µM UF        | 0.4 ± 0.02                               |
| 4                      | None            | 0.4 ± 0.03                               |

Cells (1.7 x 10^5 cells/ml) were incubated with either [125I]-labeled UF or [125I]-labeled-aglyco-UF (1 x 10^6 dpm; 0.37 µg) for 60 min. Incubations were carried out at either 37°C or 4°C (on ice). Unlabeled, glycosylated UF, was included in one set of incubations at 37°C. Each determination was carried out in triplicate. Cell-associated radioactivity was determined as described in Methods. Aglyco-UF was generated from a portion of Con A bound UF which was then digested with endo H and collected as an unbound fraction following chromatography on Con A-Sepharose.

### Figure 6. Autoradiographic localization of UF in the liver of a fetal pig following injection of [125I]-labeled-UF into the umbilical vein. A gilt was laparotomized on Day 75 of pregnancy and her fetuses exposed via an incision in the uterine wall. [125I]-labeled-UF (1.3 x 10^7 cpm) was injected into the umbilical vein. Fetuses were removed after 3 min and their livers perfused with fixatives. Pieces of liver were postfixed, sinusoidal cell (reticuloendothelial cell) and an adjacent hepatocyte sinusoids.

### Figure 7. Uptake of intact, glycosylated UF by reticuloendothelial cells in vitro (Fig. 6) and in vivo (Fig. 7) indicated that [125I]-UF was taken up by rat liver reticuloendothelial cells by a process that was specifically inhibited by unlabeled UF. In Fig. 6 the uptake of [125I]-labeled-UF was measured in presence of increasing concentrations (0.65 to 3.7 µM) of unlabeled ligand during a 15 min incubation period at 37°C. Double reciprocal plots (see insert) for two different experiments gave Kuptake values of 4 x 10^{-8} M and 3 x 10^{-8} M respectively. Addition of unlabeled UF or incubation on ice again reduced uptake of intact [125I]-labeled-UF. However neither treatment affected the accumulation of aglyco-UF.

### Figure 8. Effect of increasing concentrations of UF on the uptake of [125I]-labeled-UF by reticuloendothelial cells from rat liver. Incubations were carried out for 15 min at 37°C in Krebs-Ringer phosphate containing 0.12 (w/v) bovine serum albumin. Each tube contained 6 x 10^6 cells, 2.5 x 10^6 cpm [125I]-labeled-UF (specic activity of 2.7 x 10^7 dpm/pg) and 0, 2.5, 6.25, 12.5, 42.5 or 100 µg of unlabeled UF. Incubation volume was 0.3 ml and all determinations were made in triplicate. At the end of the incubation period, tubes were placed on ice, 1 ml cold buffer was added and cells resuspended by centrifugation (15,000 rpm, 3 min). The supernatant solution was aspirated from the pellet, and washing procedure repeated. Radioactivity associated with the cell pellet was determined using a gamma counter. The inset shows a double reciprocal plot of total uptake versus UF concentration derived from these data. Total uptake UF associated with the cells was corrected for nonspecific binding. Kuptake calculated from the double reciprocal plot, was 3 x 10^{-8} M.
Inhibitors of UF uptake. The results of several different experiments using a variety of potential inhibitors and competing ligands for uptake of UF by rat liver reticuloendothelial cells are reported in Table 5.

Table 5. Uptake of [111m]labeled-UF by rat liver reticuloendothelial cells in presence of a series of potential inhibitors.

| Additions | Concentration of Additive | Uptake of Additive |
|-----------|--------------------------|--------------------|
| None (37°C) | - | 100 |
| None (4°C) | - | 33.5 |
| None (4°C) | - | 20.6 |
| Yeast mannann | 0.4 mg/ml | 61.7±9.7 |
| Yeast mannann | 0.8 mg/ml | 34.7 |
| Ovalbumin glycopeptide | 0.13 mg/ml | 45.8±0.7 |
| Ovalbumin glycopeptide | 0.17 mg/ml | 61.7±2.4 |
| Endo-EEA | 20.6 ρM | 34.7±4.1 |
| D-mannose-biose serum albumin | 5.6 ρM | 23.2±1.9 |
| D-fucose-biose serum albumin | 5.6 ρM | 21.7±1.2 |
| D-galactose-biose serum albumin | 3.5 ρM | 69.4±5.2 |

Rat reticuloendothelial cells were incubated with [111m]labeled-UF for 40 min at 37°C (except where indicated). In each experiment, a constant number of cells (usually between 1 x 10⁶ and 8 x 10⁶/ml) were employed. The amount of [111m]labeled-UF added was also constant for individual experiments (usually between 5 x 10⁶ dpm and 2 x 10⁶ dpm). However, because of variations in cell number and other conditions between experiments, all results are expressed as percent control value (± standard error of the mean, S.E.M.) for any particular experiment. Concentrations of glycopeptides are in relation to the bovine serum albumin and not to the number of sugar residues (around 46 per molecule).

Because the cells used in the different experiments varied in number and in their ability to accumulate UF, all results are presented as a percent of the uninhibited control values obtained at 37°C for that experiment. Uptake of UF was reduced at 25°C and 4°C compared to 37°C. Selenious EDTA also inhibited uptake effectively. However, other proteins, including bovine serum albumin, transferrin and the strongly basic protein, glycoprotein, did not inhibit (data not shown). Yeast mannann reduced uptake, as did a crude preparation of glycopeptide prepared from Pronase digestion of ovalbumin. The glycopeptides, L-fucose-biose serum albumin and D-galactose-biose serum albumin, were effective inhibitors of UF uptake. By contrast, D-galactose-biose serum albumin was a poor competing hapten.

Uptake of [111m]labeled-UF by reticuloendothelial cells isolated from day 15 fetal pig liver. The uptake of UF by a population of reticuloendothelial cells isolated from fetal pig liver has also been examined (Table 6). These cells were prepared using identical techniques to those employed with the adult rat.

Table 6. Uptake of [111m]labeled-UF by reticuloendothelial cells of fetal pig liver in presence and absence of a series of potential inhibitors.

| Additions | Concentration of Additive | Uptake of Additive |
|-----------|--------------------------|--------------------|
| None (37°C) | - | 231+49/7 |
| None (4°C) | - | 999±1051 |
| Uteroferrin | 0.01 mg/ml | 117±5.9 |
| Uteroferrin glycopeptide | 0.32 mg/ml | 111±7.6 |
| Ovalbumin glycopeptide | 0.32 mg/ml | 117±5.9 |
| Ovalbumin | 0.01 mg/ml | 212±3.2 |
| Yeast mannann | 2.0 mg/ml | 212±3.2 |

A day 15 pig fetus was exposed via an incision in the uterine wall as described by Keen and others (13). The fetal liver was prepared with colleagues in an identical fashion to that described for adult rats (14). Reticuloendothelial cells were isolated by centrifugation and suspended in Ficoll-Hypaque before containing of 0.15 (mor) bovine serum albumin. Viability, determined by trypan blue exclusion, was greater than 90%. Incubation was carried out at 37°C for 10 min. These contained 1 x 10³ cells/ml. 1 x 10⁶ dpm [111m]labeled-UF (specific activity of 1.9 x 10⁶ cpm/mg).

The uptake of [111m]labeled-UF by fetal cells was approximately the same as that found with adult rat reticuloendothelial cells. Uptake was inhibited by yeast mannann and by the addition of glycopeptide prepared from ovalbumin or UF.