The physiological significance of transferrin microheterogeneity

An interpretation of the role of N-linked glycans
in transferrin and iron metabolism.

De physiologische betekenis van de microheterogeniteit van transferrine

Een interpretatie van de rol van eiwitgebonden oligosaccharides
in transferrine- en ijzermetabolisme.

Proefschrift

ter verkrijging van de graad van doctor aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus Prof. Dr. P.W.C. Akkermans M. Lit.
en volgens het besluit van het College van Dekanen.

De openbare verdediging zal plaatsvinden op woensdag 1 december 1993 om 15.45 uur.

door

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Geboren te Rotterdam

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The studies described in this thesis were carried out in the laboratory of the Department of Chemical Pathology (Head of department Prof. Dr. H.G. van Eijk), Erasmus University Rotterdam, The Netherlands.

aan mijn ouders,
aan Toos

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Preface

1. Aim of the study.

The starting point for this thesis was the observation that when attempts are made to separate monoferric transferrins from diferric transferrin by isoelectric focusing, in addition to what was thought to represent the pure monoferric transferrins, a great number of additional bands are found whose presence cannot be explained by differences in iron content. Treatment of serum samples with sialidase before isoelectric focusing decreases the number of additional bands. This variability in electrophoretic behaviour can be quantified by comparing relative amounts of components as measured through crossed immunoelectrophoresis after separation by isoelectric focusing. From a screening experiment it appeared that different patterns could be obtained in a number of pathological conditions[1], which led us to further investigate this phenomenon.

2. The work.

The first step was a literature survey on what we anticipated to be the biochemical basis for the phenomenon of microheterogeneity[2,3]: the N-glycosylation of proteins. Based on this literature survey we decided to first ascertain that differences in N-glycosylation indeed underlie the microheterogeneity of transferrin. To this end amino acid and carbohydrate analyses were performed on protein samples obtained by preparative isoelectric focusing[4]. The results confirmed the above mentioned assumption and consequently also validated our speculations as to the structure of the glycans ascribed to the different microheterogeneous forms.
Extending the initial work on transferrin microheterogeneity in serum samples, we found characteristic changes in the microheterogeneity pattern in a wide variety of patient and volunteer groups. Ideas on the physiological role of modulation of N-glycosylation were developed on the basis of the direction of changes in the context of current knowledge about iron metabolism in the pathophysiological conditions explicitly or implicitly defined by these groups[5].
Testing these hypotheses was possible after we developed a method to isolate uniquely defined transferrin subfractions[6]. Singularly defined microheterogeneous forms of transferrin were then characterised with reference to the role of transferrin in iron metabolism by incubation experiments on the cells most intimately involved in iron metabolism; i.e. hepatocytes, placental membrane and bone marrow[7]. On the basis of our findings and the literature on transferrin metabolism, a hypothesis explaining both the role of microheterogeneity in iron metabolism and the mechanism of mobilisation of iron stores in pregnancy and iron deficiency and the acute phase reactions is forwarded[7].
Extending this work on structure function relationships with particular reference to N-glycosylation, we attempted step by step removal of sugar residues from glycans still attached to the protein core, but this failed because procedures yielded either unchanged transferrin or irreversibly denatured protein. Thus, an alternative approach was chosen. Since bacteria are not capable of N-glycosylation, the approach has been to induce expression of transferrin in E. coli by recombinant DNA-techniques. This work has been performed in close collaboration with us by one of our colleagues in the department, Drs. M.J. de Smit, at the Gorlaeus laboratory in Leiden, and as a result we

have been able to obtain human transferrin with its authentic amino acid sequence, but without the N-linked glycans[8,9].

3. Future prospects

Medical colleagues often wondered why I bothered for something which did not seem lack anything except clinical relevance. Entangled as I was in the intricacies of protein glycosylation I found myself unable to provide a satisfactory answer. Now studies on the biochemistry underlying microheterogeneity have given us more insights into the role of this phenomenon in health and disease, but the holy grail of clinical relevance defined as conclusions with direct implications for diagnosis or treatment may not be apparent from our work. A future path to clinical relevance lies in the exciting new proposals for the use of transferrin in cancer chemotherapy and in the use of microheterogeneity patterns as diagnostic aids. Both these applications rely on basic research; the availability of recombinant transferrins will enable us to investigate in more detail structure function relationships, hopefully create new insights in iron metabolism and transferrin metabolism, and possibly implement this knowledge in the future.

List of publications

This thesis is based on the work described in and insights gained from writing and participating in the following publications.

Articles

1. H.G. van Eijk, W.L. van Noort, G. de Jong and J.F. Koster.
Human serum sialo transferrins in diseases.
Clin. Chim. Acta 1987; 165: 141-145.

2. G. de Jong and H.G. van Eijk.
Microheterogeneity of human serum transferrin: A biological phenomenon studied by isoelectric focusing in immobilized pH gradients.
Electrophoresis 1988; 9: 589-598.

3. G. de Jong and H.G. van Eijk.
Functional properties of the carbohydrate moiety of human transferrin.
Int. J. Biochem. 1989; 21: 253-263.

4. G. de Jong, W.L. van Noort, H.G. van Eijk.
Carbohydrate analysis of transferrin subfractions isolated by preparative isoelectric focusing in immobilized pH gradients.
Electrophoresis 1992; 13: 225-228.

5. G. de Jong, J.P. van Dijk and H.G. van Eijk.
Critical review: The biology of transferrin.
Clin. Chim. Acta 1990; 190: 1-46.

6. G. de Jong, W.L. van Noort, R.A. Feelders, C.M.H. de Jeu-Jaspars and H.G. van Eijk.
Adaptation of transferrin protein and glycan synthesis.
Clin. Chim. Acta. 1992; 212: 27-47.

7. G.de Jong, C.M.H. de Jeu-Jaspars, M.J. Kroos, W.L. van Noort and H.G. van Eijk.
In vitro binding characteristics and iron donating properties of microheterogeneous forms of transferrin.
Paper submitted.

8. M.J. de Smit, G. de Jong, J. van Duin, H.G. van Knippenberg and H.G. van Eijk.
Optimized bacterial expression of nonglycosylated human transferrin and its half molecules.
Paper submitted

9. P Hoefkens, M.J. de Smit, W.L. van Noort, H.G. van Eijk and G. de Jong.
Isolation of human transferrin from E.coli under denaturing conditions. Confirmation of authentic amino acid sequence by amino acid analysis. Restoration of solubility in aqueous solution via primary folding of the reduced recombinant protein with subsequent regeneration of intramolecular disulphides is accompanied by ability to bind iron.
Paper submitted.

10. L.J. Mostert, G. de Jong, J.F. Koster, H.G. van Eijk.
Iron mobilization from isolated hepatocytes.
Int. J. of Biochem. 1986; 18: 1061-1064.

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Transferrine-subfracties in patientensera. Fysiologische betekenis?
Ned. Tijdschr. voor Klin. Chemie 1987; 12: 259-262.

12. G. de Jong en H.G. van Eijk.
De biochemie van glycanen in glycoproteinen - met als voorbeeld transferrine.
Analyse 1991; 46: 92-95.

13. G. de Jong en H.G. van Eijk.
De biochemie van glycanen in glycoproteinen.
Tijdschrift van de Belgische Vereniging van Laboratorium Technologen. 1991; 18: 211-220.

14. R.A. Feelders, G. Vreugdenhil, G. de Jong, A.J.G. Swaak and H.G. van Eijk.
Transferrin microheterogeneity in rheumatoid arthritis. Relation with anemia of chronic disease, disease activity and cytokines.
Rheum Int. 1992; 12: 195-199.

15. H.G. van Eijk and G. de Jong.
The Physiology of Iron, Transferrin, and Ferritin.
Biological Trace Element Research 1992; 35: 13-24.

16. W.L. van Noort, G. de Jong, H.G. van Eijk.
Uit de laboratoriumpraktijk. Ideale scheiding en kwantificering van sialo-transferrinefracties in humaan serum.
Tijdschrift N.V.K.C. 1993; 18: 43-45.

17. H.G. van Eijk, W.L. van Noort en G. de Jong. Scheiding en kwantificering van iso-transferrinen. Tijdschrift van de Belgische Vereniging van Laboratorium Technologen. 1993; 20: 187-204.

18. G. de Jong, W.L. van Noort and H.G. van Eijk. In Press 1993: Optimized separation and quantitation of serum and cerebrospinal fluid transferrin subfractions defined by differences in iron saturation or glycan composition. Progress in Iron Research, editor C. Herschko, Plenum Press, New York.

Abstracts

1. L.J. Mostert, G. de Jong, H. v. Dorst, J.F. Koster and H.G. van Eijk. Iron chelators, new chelators. Proc. 27th Dutch Federation Meeting, Groningen, april 2-3, 1986.

2. G. de Jong, W.L. van Noort, H.C.S. Wallenburg and H.G. vam Eijk. Transferrin microheterogeneity during pregnancy and the use of oral contraceptives. Proc. 29th Dutch Federation Meeting, Utrecht, april 6-8, 1988.

3. G. de Jong, W.L. van Noort, M.J. Kroos and H.G. van Eijk. Physiological patterns in the glycosylation of human transferrin and functional characteristics of microheterogeneous forms of transferrin. European Iron club meeting, Frankfurt am Main, sept 5-8, 1988.

4. G. de Jong, N. de Jeu-Jaspars, M.J. Kroos and H.G. van Eijk. A note on the influence of transferrin microheterogeneity of human serum transferrin on iron uptake and transferrin binding by HepG2-cells and syncytiotrophoblast membrane, respectively. Proc. 30th Dutch Federation Meeting, Maastricht, march 29-30, 1989.

5. G. de Jong, N. de Jeu-Jaspars, M.J. Kroos and H.G. van Eijk. Hepatocyte iron uptake and transferrin microheterogeneity. European Iron Club meeting, Budapest, aug 28-31, 1989.

6. G. de Jong, R.A. Feelders, W.L. van Noort and H.G. van Eijk. Transferrin microheterogeneity in iron deficiency anemia. Abstract European iron club meeting 1990, Lissabon of Porto.

7. G. de Jong, C.M.H. de Jeu-Jaspars, R.A. Feelders, W.L. van Noort and H.G. van Eijk. Adaptation of transferrin protein and glycan synthesis. International Meeting on Porphyrin Metabolism and Iron Metabolism, apr 30th - may 4th 1992, Papendal, the Netherlands. Neth. J. Medicine, 1993, vol 42, p. A37.

Chapter 1.

General introduction

Transferrin in the context of iron homeostasis

Nomenclature and historical perspective

General Considerations

Transferrin in the context of iron homeostasis

The earth's core consists for the major part of iron, and amongst metals in the earth's crust it is second only to aluminum[1]. As a result, abundant supplies of both ferrous and ferric iron are present in the biosphere, which is suitable for iron is an absolutely essential nutrient for virtually all forms of life examined (the lactobacillus is one of the very few organisms known not to require iron for growth[2]). Paradoxically, despite the propitious geological starting point, without exception organisms have to rely on ingenious methods for uptake and transport of iron. This is due to the fact that in the present oxidizing atmosphere in aqueous solutions Fe(III) is the thermodynamically favoured iron species. The consequent physiological conditions complicate the availability of iron to both plant and animal cells, i.e. the extremely low solubility of ferric ions in oxygenated fluids at neutral pH (10^{-17} mol/l) and the fact that cell membranes are essentially impermeable to iron in its trivalent state. Hence, for the transport of iron in both aqueous and lipid phases special requirements must be met. The solutions acquired by different species involve organic chelates such as ferric citrate, or for plants ferric galate, and for bacteria siderophore chelates. In animals, and this also applies to a few parasitic protozoa and bacteria, transferrin and possibly also ferritin supply the bulk of the iron to the cell surface. Little information on the nature of the transmembranous and intracellular transport vehicles is available. Current research points to an important role for low molecular weight fractions, notably physiological cell components such as ATP and ascorbate[3,4], but their concomitant or consecutive roles in transport to and from iron binding proteins and iron containing enzymes have not been clarified.

Once intracellular storage is remarkably uniform. From bacteria through fungi to the animal and plant kingdoms, iron is stored in its ferrrous form as a polynuclear $(FeOOH)_8(FeO^-OPO_3H_2)$ - core within a protein shell composed of a number of ferritin molecules[5]. In this form the organism is protected from participation of the ferrous iron in toxic oxidation-reduction

reactions, but remains capable of mobilising these iron stores on demand.
Most of the recent progress in the understanding of iron metabolism has been derived from advances in the research of the iron binding proteins ferritin, lactoferrin and transferrin. In this thesis my effort to contribute to the understanding of transferrin and iron metabolism is described.

Nomenclature and historical perspective

The transferrins form a group of evolutionary related proteins. Members of the group (synonyms between brackets) are:

1. Transferrin (siderophilin), a metal-binding ß-globulin of vertebrate blood plasma and other extracellular fluids, such as milk, semen, amniotic fluid, cerebrospinal fluid (tau-protein, myotrophic factor; sciatin). Transcripts from the same gene are also found in bird and reptile oviduct secretions and egg whites (ovotransferrin or conalbumin).
2. Lactoferrin (lacto-transferrin) of mammalian extracellular secretions (milk, pancreatic juice, tears) and intracellular in leukocytes.
3. Malignancy associated transferrins and pseudogenes : p97, an integral membrane protein of human malignant melanoma cells and some foetal tissues (melanotransferrin, genuine transferrin), chicken lymphoma ChBlym-1 transforming protein, Burkitt lymphoma transforming protein.
4. Invertebrate iron binding proteins in the blood of some crustaceae and urochordates.

This somewhat confounding nomenclature of these iron binding proteins is best understood in the light of the history of iron and transferrin research.
The use of iron-containing substances to relieve the symptoms of anemia has been an established empiric fact dating back to pre-Egyptian history. The first scientific facts on the role of iron in living organisms were registered in 1713 by Lemmery and Geoffroy who demonstrated the presence of iron in animal tissues[6]. In 1843 J. Liebig published his book *Tierchemie*. In the chapter on respiration he proposed that all oxidation of biological substrates occurred in the blood; in his view the iron contained in haemoglobin played a central role in this process. This view was adopted by many of his contemporary scientists, amongst others by Hoppe-Seyler who was the first to isolate haemoglobin in purified crystalline form in 1867. After microspectroscopic examination of slices of animal muscle tissue under oxidizing and reducing conditions, McMunn in 1866 forwarded the view that all tissues contained pigments that were involved in cellular respiration implying that haem-containing proteins were not restricted to erythrocytes alone, but present in all living cells[7]. Conflicting with the intravascular oxidation theory of Liebig his view was sharply criticised by Hoppe-Seyler and his followers in the years after, with the result that McMunn's hypothesis was discarded. Unintentionally, in perpetuating the idea that all iron in blood was contained in haemoglobin this also blunted initiatives to rejuvenate research into the nature of iron found in plasma.
Advances in the understanding of cellular respiration initiated by the work of Otto Warburg around 1910 (" Eisen, den sauerstoff-übertragenden Bestandteil des Atmungsferment") and the rediscovery of Mc Munn's findings by Keilin with the demonstration of iron in the haem-containing proteins that became known as cytochromes in cells other than erythrocytes in the 1920's, not only eventually lead to the conception and elucidation of the structure of the

respiratory chain but also reopened the discussion on iron transport in the body.
In 1925 Fontès and Thivolle demonstrated that iron in blood plasma was not identical to iron in haemoglobin[8], a finding for which they coined the term *fer circulant*. In 1927 this was corroborated by Barkan[9] who added to this the notion that this iron was bound to a substance of high molecular weight from which it could not easily be separated at physiological pH by dialysis. On the other hand the iron could easily be separated by mild acidification (as opposed to the more brusque methods needed to separate iron from intracellular haem-proteins), and in turn he named it *das leicht abspaltbares Bluteisens*. In 1937 Barkan and Schales were able to establish that the high molecular substance involved was a globular protein[10] (incidentally both articles were published in *Hoppe-Seyler's* Zeitung für Physiologische Chemie).
This high molecular weight substance was considered to play an important role in iron metabolism, but unequivocal correlation of the limited laboratory findings with clinical conditions was not possible. Interested in this subject L. Heilmeyer and coworkers investigated therapeutic effects of iron salts in anemia from 1936 on[11], and in 1945 Holmberg and Laurell tried to correlate the iron binding capacity of serum with iron transport[12], but evidence for the importance of transferrin in iron metabolism was difficult to obtain. Heilmeyer was rewarded for his longstanding interest in iron metabolism in 1961 with the discovery of a patient suffering from a severe anemia with the characteristics of iron deficiency despite having high liver iron stores (and repeated infections). This patient provided what is still simply the ultimate certification of the importance of transferrin in iron homeostasis (and health in general), for this was the first patient identified as suffering from the extremely rare condition of atransferrinemia[13].
The nomenclature for iron binding proteins in body fluids has evolved from early clinical chemistry. The first iron binding protein of this class was detected in milk in 1939. This protein was later shown to be produced not only by epithelia in lactating glands but also by epithelia producing sweat, tears, saliva and pancreatic juice and to be present intracellularly in leucocytes, particularly increasing in acute phase responses. This protein has been named *lactoferrin* and is a protein with many structural similarities to transferrin, but not identical to it.
In 1944 Schade and Caroline observed an inhibitory influence of an iron binding protein isolated from eggwhite on the growth of Shigella species[14]. This eggwhite protein was called *conalbumin* and later shown to be identical to *ovotransferrin*. Recently it has been established that both liver and oviduct transferrin are transcribed from the same gene. Regulation of gene transcription in these tissues is dissimilar, the endproduct is nevertheless an identical polypeptide. Finally, in 1946 Schade and Caroline were able to demonstrate similar bacteriostatic properties for an iron binding protein isolated from plasma[15]. This protein isolated from plasma they named *siderophylin*. Although one could argue that in the light of current understanding this is a more appropriate name, it was the name forwarded by Laurell and Holmberg in 1947[16] that has become the most commonly used, probably because it reflects its role in iron metabolism more explicitly: *transferrin*.
The latest intriguing addition to the transferrin family is the P 97, also known as melanotransferrin since it was first identified in a malignant melanoma-derived cell line. This protein has one characteristic that sets it apart from the other transferrins: it is an integral membrane protein. Moreover, despite elaborate efforts[17], to date an active role in iron transport has yet to be identified for this protein.

General Considerations

Transferrins are monomeric glycoproteins found in the biological fluids of invertebrates and vertebrates, with the property of reversibly binding iron[18,19]. Transferrin is believed to regulate iron-fluxes between sites of absorption, storage and utilisation, the most important in the last respect being the bone marrow. Full understanding of the functions of transferrin has not been achieved. Its presence in culture media is probably essential for the growth of cultured cells that do not synthesise transferrin themselves[19-21]. This indispensability of transferrin may extend to all chordates, since invariably animals belonging to three of the classes of the Phylum Chordata (Reptilia, Aves and Mammalia) have been shown to carry transferrins in their blood, or comparable body fluids[22]. Also primitive vertebrates such as the lamprey[23], the hagfish[24,25] and the tench[26], have monomeric transferrin-like molecules with two iron binding sites and a molecular mass of approximately 78,000 Dalton. The Lamprey *Geotria australis* Gray has a tetrameric form made out of four 78,000 Dalton subunits with two iron-binding sites per subunit[27]. Xenopus species have also been shown to have transferrin-like molecules with a molecular weight of about 85,000 Dalton[28]. Even in cases of so-called atransferrinemia, trace amounts of transferrin can be detected[29,30], suggesting that absolute atransferrinemia is incompatible with life. It is not yet clear which function of transferrin is responsible for this indispensability-phenomenon. In some culture systems it appears to be independent of the iron donating capacity of transferrin. Conceivably this is related to the recently described interaction of transferrin with the transplasmalemma NADH reductase (chapter 4).

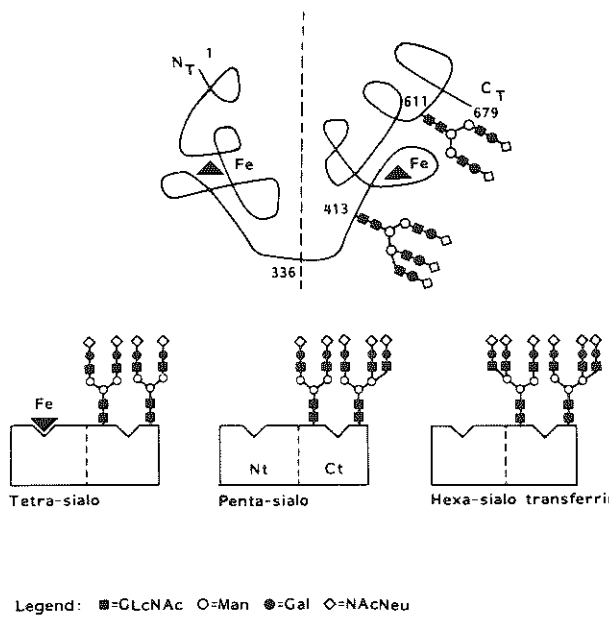


figure 1. Schematic representation of the transferrin molecule carrying two N-linked glycans in the C-terminal domain. Three microheterogeneous forms of human transferrin are shown at the bottom of the figure.

In humans, transferrin is one of the most extensively studied plasma proteins. The aminoacid sequence of human serum transferrin, the localisation of the gene (q21-25, long arm chromosome 3) and many of its structural features including the complete base sequence, have been established[31-34]. Minor differences exist between the aminoacid sequence predicted from the nucleotide sequence of cDNA probes and the chemically determined aminoacid sequence, which is a usual finding when comparing results of dissimilar sequence analysis techniques. Most of the differences relate to the existence or absence of amidation of side chains, but none of the differences has direct implications on secondary structural features such as disulphide bridges and glycosylation sites.

Human transferrin consists of a single polypeptide chain containing 679 aminoacid residues carrying two N-linked complex type glycan chains. For apotransferrin the calculated molecular mass is 79,570 Dalton, of which 4,412 Dalton (5,5 %) can be accounted for by two complex type biantennary glycans. Some physicochemical data on transferrin are given in Table I.

Table 1. Aminoacid composition of human serum transferrin and physicochemical properties[32,33,35]. The amino acid values have been based on the cDNA characterization by Yang et al[31].

Aminoacid	mol/mol
Lysine	58
Histidine	19
Arginine	26
Aspartate	45
Threonine	30
Serine	41
Glutamate	43
Proline	32
Glycine	50
Alanine	57
Cysteine	38
Valine	45
Methionine	9
Isoleucine	15
Leucine	59
Tyrosine	26
Phenylalanine	28
Asparagine	34
Glutamine	16
Tryptophan	8
Total	679

Property	Apotransferrin	Diferric Transferrin
Molecular Mass	79,570	79,898
Sedimentation constant S° 20,w. (± 0.2)	5.4	5.16
Stokes radius	3.53 nm	3.58 nm
Intrinsic viscosity (ml/g)	4.10 ± 0.15	4.05 ± 0.15
Axial ratio (prolate ellipsoid)	2.5	2
Hydrated Volume(cm^3)	15.4 x 10^{-20}	16.9 x 10^{-20}
Molar absorption coefficient 280 nm	88,200	114,000
470 nm	-	4620
Isoelectric points (4-sialo Tf C_1)	5.80	5.45
E_0 Fe(II)/Fe(III)		-310 mV
Iron binding sites:		
Number	2	
K_a (pH=7.4)		
N-terminal domain	$1 \cdot 10^{22}$ M^{-1}	
C-terminal domain	$6 \cdot 10^{22}$ M^{-1}	

The transferrin molecule can be divided into two homologous domains, the N-terminal domain (residues 1-336) and the C-terminal domain (residues 337-679) with the carbohydrate moieties in the C-terminal domain at positions 413 and 611 (Figure 1). The presence of two domains with a high degree of internal homology (up to 40%) in the transferrin molecule has

been demonstrated by partial proteolysis of a number of transferrin species, producing fragments containing about half the polypeptide and a single metal-binding site[36-38] and by aminoacid sequence studies[32]. Low resolution X-ray crystallographic studies of rabbit serum transferrin have shown that the polypeptide chain is folded into two globular domains[39]. These observations were extended by 0.32 nm X-ray diffraction-analysis of the iron saturated human lactoferrin[40]. This study showed that the N- and C-terminal halves form two separate globular lobes, each of which has two segments surrounding a cleft. At the base of these clefts, hidden below the surface of the molecule, the specific iron binding sites are located.

A comparable situation is thought to exist in human serum transferrin. The molecule is divided in two globular domains, each containing one metal-binding site, binding a ferric ion with a K_a of approximately 10^{22} M^{-1}. The concomitant binding of an anion (physiologically bicarbonate) is essential for metal binding at each site. Although iron is the most important metal bound by transferrin *in vivo*, transferrins are capable of binding over 20 metal ions including Cr^{3+}, Cu^{2+}, Mn^{2+}, Co^{3+}, Co^{2+}, Cd^{2+}, Zn^{2+}, Ga^{3+}, Ni^{2+}, Al^{3+}, Sc^{3+}, In^{3+}, and Gd^{3+}, albeit that the affinity for most of these ions is less than that for Fe^{3+}. Nevertheless in the circulation trace elements such as aluminum and zinc are located predominantly in the transferrin fraction, and as such transferrin plays an important role in the homeostasis of these elements.

The two iron binding sites are virtually independent since the affinity of either site for iron changes little with occupancy of the other. This does not mean that there is no biophysical difference between the binding sites. At pH 6.7, the affinity for iron at the binding-site in the N-terminal domain is less than one-twentieth the affinity for iron the binding-site located in the C-terminal domain. Hence the binding site in the C-terminal domain has been termed the acid-stable site. Under conditions prevailing in the circulation, the effective affinity constants of the N- and C-site amount to 1 and 6 x 10^{22} M^{-1} respectively[41,42]. Recently these parameters have been extended in the pH-range physiological in the endosomes involved in receptor mediated endocytosis. The denominations acid-stable and acid-labile sites of receptor-bound transferrin at that pH are incorrect since it is the N-terminal domain displaying the higher affinity then, a fact that highlights an additional role for the receptor itself in iron transport (see paragraph on iron uptake, chapter 4).

The bilobed structure together with the existence of a high degree of internal homology between the two domains has led to the hypothesis that all modern, vertebrate (bilobed) transferrins arose during the course of evolution by duplication and fusion of a gene specifying a simpler single-domain protein. This gene-duplication hypothesis is supported by a number of other structural features. The chicken ovotransferrin gene and the human transferrin gene display a high degree of homology between pairs of exons of the individual genes and similarities in splicing patterns in corresponding exon pairs[31,33,43], consistent with the proposed gene duplication. Also, as suggested by Williams, the conservative disposition of the disulphide bridges reinforces this idea[44].

Two theories on the characteristics of the archetypal unilobed transferrin later involved in the gene duplication have been proposed. In both theories the duplication of this primordial gene offers the biological advantage of production of a bilobed, enlarged transferrin molecule. This enlargement prevents loss of transferrin through glomerular ultrafiltration with loss of both iron and protein through urinary excretion. Indeed, isolated domains of hen transferrin injected into the circulation of mice are rapidly excreted in the urine[45].

In the first theory a freely circulating single lobe transferrin is envisioned to have been the predecessor. Such a molecule has been claimed to exist in a primitive urochordate[46]: An iron-binding protein with a molecular mass of about 41,000 Dalton and one iron binding-site,

capable of donating iron to rat reticulocytes through binding to the transferrin receptor and subsequent endocytosis. The urochordate referred to, *Pyura stolonifera*, allegedly lacked the evolutionary drive to exclude the mono-sited ancestor because this animal does not possess a filtration kidney and is thus capable of holding a transferrin of smaller molecular size. Whether this substance is truly a transferrin is questionable, since one of the pertinent characteristics of all bilobed transferrins, the concomitant binding of a bicarbonate ion upon the binding of a ferric ion is lacking in this protein (P. Aisen, personal communication).

Williams has postulated that the ancestral transferrin gene may have arisen in urochordates as a cell membrane-anchored 40,000 dalton protein[44]. As indicated above sustained existence in the circulation would be impossible. Therefore the anchorage may have enabled it to serve as a membrane-associated iron receptor. The membrane bound tumour antigen P97 is homologous to transferrin and binds iron. P97 can thus be viewed as the rudimentary witness of that evolutionary stage where duplication of the gene had already occurred, but before the protein had gained entrance to the blood stream. Other characteristics of the melanotransferrin that would argue in favour of this proposition are the highest internal homolgy of all transferrins (46 % as opposed to values ranging from 33 to 41 %), the location of its gene also on chromosome 3, and the 37 - 39 % sequence homology with human serum transferrin, lactoferrin and chicken transferrin. However, even though a large number of membrane iron binding sites were identified in the melanoma derived cell line that expresses the melanotransferrin on its surface (387,000 per cell), and although the melanotransferrin is capable of binding ferric iron offered to it in the form of ferric citrate, intracellular uptake from this membrane bound iron pool could not be detected[17].

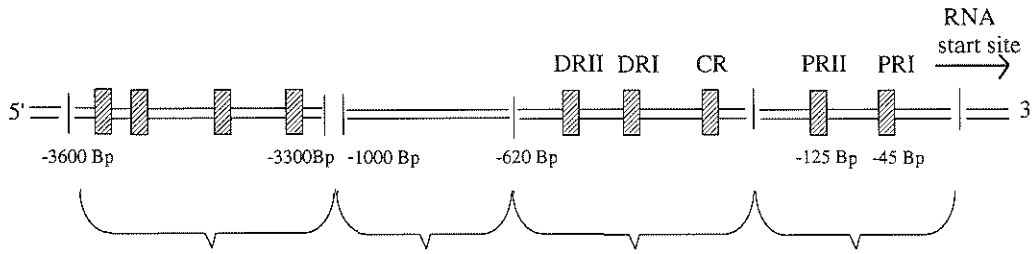
Thus, the gene-duplication theory as such is generally appreciated, but the question of the origin of the bilobed transferrin remains.

Chapter 2.

Control of transferrin synthesis

The principal site of synthesis of transferrin that enters the circulation is the hepatocyte. Other cells shown to constitutively produce transferrin are Sertoli cells, brain capillary endothelial cells, ependymal cells in the choroid plexus, and oligodendroglial cells in the brain. During lactation, from a quantitative point of view, the mammary gland is also an important transferrin source. From a qualitative point of view transferrin production in testis and brain is interesting. By virtue of respectively the haematotesticular and the blood brain barrier in these organs a distinct extracellular environment is created. Despite the lack of high iron requirements, transferrrin is still an essential element in these compartments, explaining the need for local production of transferrin albeit at slower rates than in the liver (estimated at one tenth the rate in the liver). This tissue-specific expression is the result of the interaction of different sets of nuclear binding proteins with cis-acting elements in the transferrin gene in each organ[47-49].
Tissue-specific transferrin gene expression inevitably starts at and discloses itself through the fetal and embryonal stages. During rat embryogenesis, transferrin has been shown to be synthesized by a variety of embryonic and extra-embryonic cells. The visceral yolksac is the major source of transferrin in the early gestational period. Later on, the foetal liver shows the highest level of Tf-mRNA expression. Tf-mRNA can also be detected in spleen and kidney of the rat embryo[50,51]. This temporal control during development is further developed after birth. Transferrin synthesis in kidney, muscle and spleen progressively diminishes after birth. In the brain and testis transferrin mRNA-levels are very low in the fetal stages but increase significantly after birth, eventually reaching about 10 % of the level attained in liver tissue, and this is maintained throughout adult life. Although it has not been possible to test this proposition in brain tissue, the idea is that in these particular organs transferrin is one of the components that are added by these matrix-defining cells to the microenvironment that is essential for normal function of these organs. This is derived from the observation that the beginning of spermatogenesis coincides with the detectability of transferrin in the testis following production of transferrin mRNA in Sertoli cells and the fact that normal differentiation of germinal cells depends on the presence of transferrin, indicating that the ability to synthesise transferrin becomes restricted to certain cell types depending on the particular developmental pathway. As Zakin noted[49] this does not imply that tissue-specific expression of transferrin is restricted to one embryological origin. Hepatocytes arise from endoderm, Sertoli cells from the mesoderm and nervous cells from the ectoderm, implicitly illustrating the importance of transferrin to the organism at all levels.
The concentration of serum transferrin is regulated by a complex interplay of factors.Increased synthesis can be realised at transcriptional as well as at translational levels.

5' flanking sequence transferrin-gene:



Enhancer element consisting of 2 domains	**Distal negative acting region**	**Distal Promotor**	**Proximal Promotor**
Acts by blocking the effect of the negative acting element downstream. **Domain A:** 1 binding site 2 liver specific nuclear binding proteins identified with affinity for this region known: HNF3α 45 kDa protein **Domain B:** 4 binding sites 3 binding proteins discerned binding to these 4 sites: NF1; affinity for two sites AP4 Unidentified protein Enhancer not active in Sertoli cells	Deletion causes upregulation of mRNA producting; mechanism unknown.	Regulates proximal promotor activity. 3 binding sites identified	Regulates tissue specificity 2 Binding sites identified: PR I & PR II Nuclear binding proteins tissue-specific: Liver: HNF-4 binds to PR I EBPα binds to PR II Nuclear binding proteins in Sertoli cells unknown but definitely distinct from liver proteins for both regions

Figure 2. Current view on regulatory principles in transferrin gene transcription. Tissue specific transcription is defined by the existence of particular subsets of DNA-binding proteins in particular cell lineages. Levels are further adapted through DNA-binding protein-dependent modulation of enhancer activity which is the result of interactions in distal promoter and the far distal enhancer element. Tf mRNA-levels in Sertoli cells for instance being 1/10 th of liver levels due to the lack of blockage of distal negative acting element. Since low levels of transferrin are detected in the plasma of individuals with so called atransferrinemia, this affliction may be related to alterations in either the distal enhancer or the distal negative acting region consequently blocking hepatic enhancement of basal production levels.

The concentration of transferrin in human serum is increased during iron-deficiency anaemia and pregnancy, and is decreased in hemochromatosis. Steroid hormones have been shown to stimulate Tf-mRNA and transferrin protein synthesis in chick liver and oviduct and in rat liver[51-53], while at the same time Tf-mRNA levels in brain, testis, spleen and kidney were not enhanced. In nutritional iron deficiency mRNA levels increased in chicken and rat liver, but not in oviduct. Catabolism of serum transferrin did not change in iron deficiency. Iron overload did not influence transferrin gene transcription.
In contrast, Tuil and co-workers were unable to demonstrate a stimulatory effect of either castration, estradiol administration or iron deficiency in the rat[54]. They suggested that the regulation of transferrin concentration in serum in response to an altered body iron status is not primarily a transcriptional event. Since no overt methodological differences are described, no explanation for this disagreement is available. Metal responsive elements in the promoter region of the transferrin gene have been shown to exist[34.55.56], but the mechanisms involved in transcriptional and translational regulation of the transferrin synthesis in response to iron deficiency have not been fully elucidated. With respect to regulation of cellular iron uptake the transferrin receptor may be more important, since its gene expression has been shown to be much more sensitive to intracellular iron concentration than transferrin gene expression[57-60]. The expression of the transferrin receptor gene is not directly enhanced; the effect appears to be attained through iron responsive elements in the untranslated region of the mRNA that modulate mRNA stability[61.62].
Returning to the issue of tissue-specific transferrin expression, much information has become available since the elucidation of the complete structure of the transferrin gene[33]. The gene as such is located on chromosome 3 (together with the transferrin receptor and the melanotransferrin gene). It consists of 17 exons and 16 introns preceded by regulatory sequences as described in figure 2.
Transcription being one of the central points at which eucaryotic gene expression is regulated, much research into the nature of these regulatory elements has been done. Presently, four distinct functional regions in the 5' flanking region of the gene have been identified:

1. Tissue specificity regulating proximal promotor region,
2. Distal promotor region regulating proximal promotor activity,
3. Negative acting region, downregulating transcription from the transferrin promoter,
4. Distally located enhancer element.

Since no deletion in the proximal promoter region could convert a hepatoma-specific region into one that was active in a non-hepatocellular cell line, it has been concluded that liver tissue specificity must result from a liver specific stimulus[56]. Distinct DNA-binding proteins have been shown to exist for both positive acting elements in the proximal promoter region. The distal promoter region is composed of a combination of possibly three positive and two negative acting elements. Most of them have been shown to interact with DNA-binding proteins[34.56]. A third region has been identified, distal to the promoter region. Deletion of this part of the 5'-flanking sequence gave rise to a transcriptional increase, implying a negative regulatory role for this sequence. Finally, a fourth, far distal region in the 5'- flanking region of the transferrin gene has been identified as an enhancer region. The current view on the organisation of regulatory elements involved in transferrin gene expression is given in figure 2.
Summarising the scheme, expression of the transferrin gene to a certain level is authorised by

positive acting elements in the proximal promoter region in response to tissue specific DNA binding proteins. Through the expression of these proteins, tissue specificity of transferrin gene transcription is regulated. This provides certain cell types with a basal level of expression that is further modified by the interaction of both tissue specific and ubiquitous DNA-binding proteins with distal promotor and enhancer regions, that ultimately define the tissue-specific level of transferrin gene transcription.

A total of nine elements distributed over four regions in the 5'-flanking region of the transferrin gene have presently been implicated in transferrin gene expression. Simultaneous interaction of a number of nuclear binding proteins is a general mechanism required to ensure high specificity of the transcription control signal as nonspecific binding of regulatory proteins is not inconceivable in large genomes. The relatively high number may be the reflection of the status of this gene: a highly conserved gene cautiously tuned to diverse physiological demands.

Chapter 3.

Chemical determinants of electrophoretic behaviour

First determinant

Second determinant

Third determinant

Miscellaneous factors

For many years it has been recognised that immunoaffinity-purified transferrin displays a complex electrophoretic behaviour indicating that transferrin is not a homogeneous substance. In the past years a great number of studies has elucidated the structural basis of transferrin heterogeneity and as a result three main categories of causes can now be considered (figure 3):

1. Variation in the polypeptide chain; genetically determined aminoacid substitutions or deletions.
2. Differences in iron content.
3. Differences in the structure of the N-linked glycan chains.

First determinant

The first determinant of electrophoretic behaviour, genetic polymorphism, is best revealed after neuraminidase treatment and saturation of serum with iron. Genetic polymorphism of plasma transferrin was first described by Smithies employing starch gel electrophoresis for separation[63]. At least 22 functional variants have been detected by starch or agarose gel electrophoresis. The most common phenotype (more than 95% in European populations) has been designated TfC, the more anodal (faster running) variants TfB, and the more cathodal (slower) TfD. Phenotypes within the main groups are designated by subscripts (TfD_{Chl}, TfD_1, etc.). The aminoacid substitutions in some of these variants have been determined and were shown to be single aminoacid substitutions. Each of these could be explained by a mutational transition in the second nucleotide of the involved codon[31]. Until 1978 TfC was considered to be a single variant. However using isoelectric focusing (IEF), TfC was shown to consist of two subtypes; TfC_1 and TfC_2[64,65]. Subsequently additional C-variants have been detected by IEF, the total number of C-variants is now claimed to be 16[66,67]. Also a number of null alleles have been detected in various populations[30,68-70]. In most of these cases, transferrin

synthesis is not completely blocked, suggesting distal mutations affecting the distal promotor enhancing element (figure 2).
The degree of transferrin polymorphism in most populations has therefore spuriously increased to about 29%. Although distributions in different populations vary, of all 38 or more variants only 4 occur with a frequency over 1%[67]. Most Tf-variants show the same iron binding capacity as the common type TfC. An unusual human transferrin having abnormal iron binding properties and an abnormal interaction with the transferrin receptor has been identified[71,72].

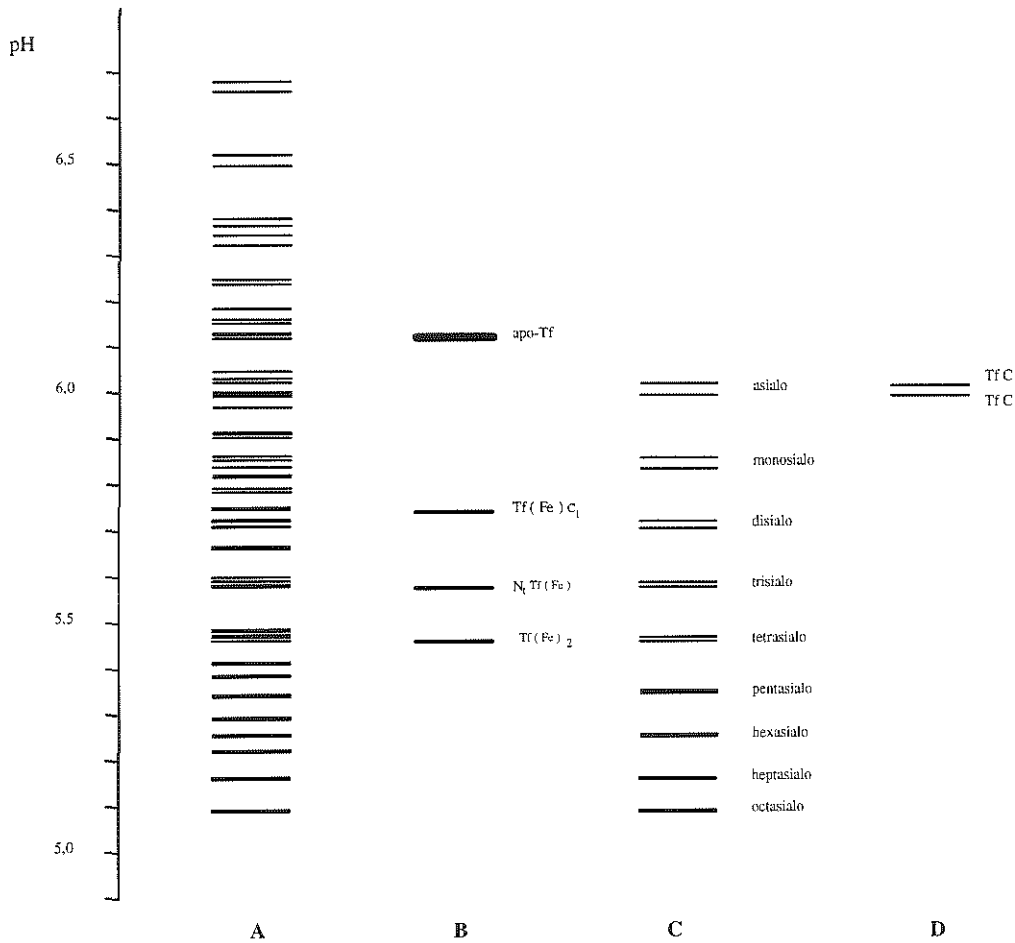


Figure 3. Transferrin microheterogeneity of an individual with the C_1C_2 genotype as in principle detectable by isoelectric focusing. (A) Total number of bands in untreated serum. (B) Major bands in untreated serum as revealed by isoelectric focusing in carrier ampholyte gels, corresponding to tetrasialotransferrins that can be separated on the basis of differences in iron content. (C) Microheterogeneous forms of transferrin that can be distinguished in an iron-saturated serum sample. (D) Reduction of the total number of 72 bands to 2 by saturation with iron and neuraminidase treatment of the serum sample, a procedure useful in the assessment of genotypic variation.

Moreover even the common C-alleles have been shown to differ in their iron binding capacities: the iron binding capacity of transferrin from subjects with the 1-1 subtype of TfC is signifi cantly higher than that of the 2-2 subtype, as well as that of the 2-1 subtype[73]. Reports about the increased prevalence of TfC_2 and TfC_3 in preterm infants and the increased frequency of the C_2-allele and especially of the C_2-type among women with a history of spontaneous abortion[74,76] have suggested the possibility that other functions than its iron carrying capacity may also be influenced by genetic polymorphism.
One might relate this to still the most striking general observation on the prevalence of transferrin variants: From Eskimos to Aboriginals, the C_1 subspecies has been identified as the oustanding predominant transferrin, suggesting a strong selectional advantage.

Second determinant

A second factor determining the electrophoretic behaviour of transferrin is the iron content. Under physiological conditions serum transferrin is approximately 30% saturated with iron. Consequently, employing techniques such as urea gel electrophoresis[77] and isoelectric focusing[78], in fresh serum four different forms of transferrin with respect to the iron content can be distinguished and isolated: apotransferrin, Tf; monoferric transferrin with iron in the N-terminal domain, Fe_NTf; monoferric transferrin with iron in the C-terminal domain, $TfFe_C$; and diferric transferrin, Fe_2Tf.
The question whether the two iron binding-sites serve different physiological purposes *in vivo*[79] is still uncertain. The predominance of Fe_NTf over $TfFe_C$ in sera from healthy donors[41,77,78,80], despite the uniformity of these transferrins in *in vitro* systems and *in vivo* with respect to their iron donating behaviour[81-83] is the basis of this uncertainty. The predominance of Fe_NTf over $TfFe_C$ in serum is a generally accepted concept. However, one group of researchers has unremittingly disputed the non random distribution of iron among the two binding-sites of serum transferrin in both healthy individuals[84] and patients with haematological disorders[85].
Finally, although those in favour of the non-random distribution by far outnumber those denying it, it may be that the controversy lingers on simply because all groups have been studying an artefact. This is considered in the light of the observation that the distribution of iron can be altered by storage of serum samples at -80°C[86]. Confirming this, we have observed a shift from non random to random distribution upon prolonged storage of serum samples at -20 °C.
An essential feature with respect to the role of transferrin in iron metabolism is the fact that it has been shown that the transferrin receptor has greater affinity for diferric transferrin than for either monoferric transferrins or apotransferrrin. This and the fact that apoTf has low affinity for the transferrin receptor at physiological pH and a high affinity at a low pH, are key properties in the model explaining both the recycling of transferrin after receptor mediated endocytosis and the high effectiveness with respect to iron donation of this system[87,88].

Third determinant

The third determinant of the electrophoretic behaviour of transferrin is the carbohydrate moiety.

Jamieson noted the influence of protein linked oligosaccharide structure on electrophoretic mobility in 1965. In performing starch gel electrophoresis on transferrin samples treated with neuraminidase, 4 components appear successively[89]. The normal occurrencence of such diverse forms defined by differences in glycan structure in serum samples was not be noted by him. This phenomenon, nowadays termed microheterogeneity, has become detectable after the introduction of more powerful electrophoretic separation techniques, notably the isoelectric focusing[90].

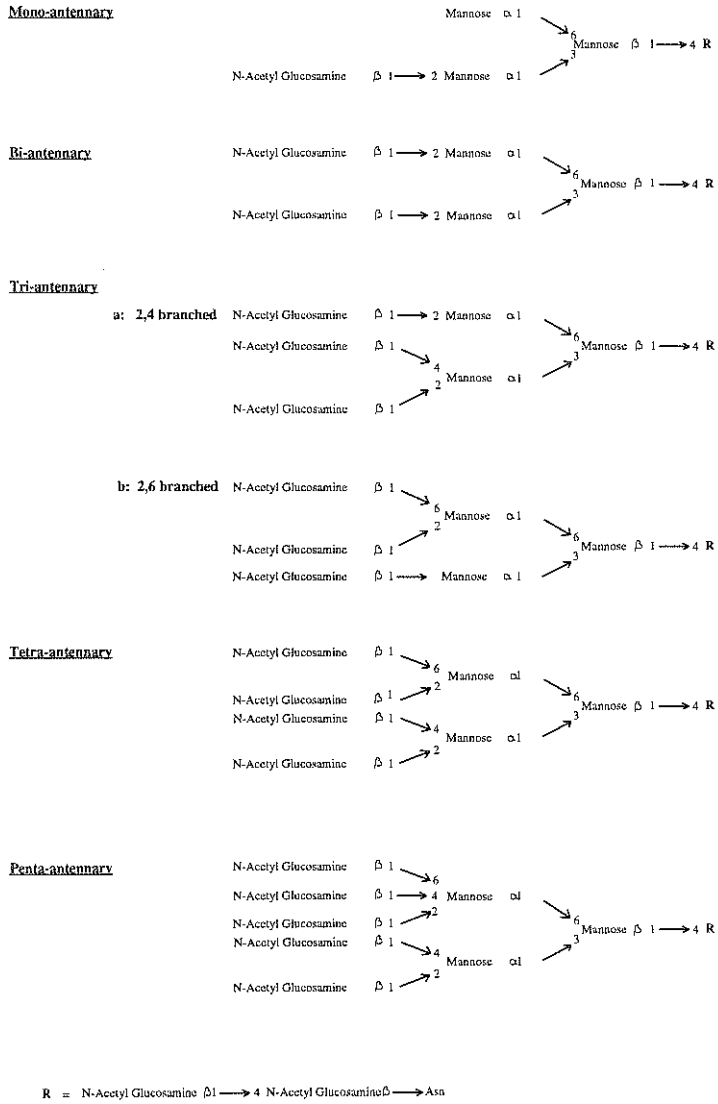


Figure 4. Branching of complex type N-linked glycans.

Transferrin carries two oligosaccharide chains in the N-terminal domain. These branched chains referred to as glycans are covalently linked to Asparagine residues. As the glycan is linked through the nitrogen atom in the R group of Asparagine these structures are referred to as N-linked glycans. Oligosaccharide chains of different composition, but in many cases also carrying sialic acid as the ultimate residue, linked through oxygen in the R groups of Serine or Threonine, also exist in glycoproteins. These so called O-linked glycans are however not found in transferrin.

The two N-linked oligosaccharide chains of transferrin have been shown to be structurally variable[91-93]. Glycans can differ in their degree of branching since bi-antennary, tri-, tetra- and even penta-antennary structures have been shown to exist (figure 4). Apart from this they can also differ in their terminal sugar residues. Glycans can be partially desialylated or even lack galactose or N-acetyl glucosamine residues. It has been proposed and accepted as an axiom that the structure of two (or more) glycans on one protein molecule was uniform (Kerkaert-Bayart proposal). This has been disproved; one molecule may carry differently branched glycans.

The biosynthetic machinery responsible for this structural diversity is interwoven with protein synthesis and in particular linked to those compartments that supply the matrix for the system involved in the synthesis of proteins that are destined to be excreted or incorporated in the cell membrane: the rough endoplasmic reticulum and the Golgi system. (For an elaborate description of the biochemical and cell biological basis of glycoprotein microheterogeneity the reader is referred to reviews listed numbers 94-98 in the references-section).

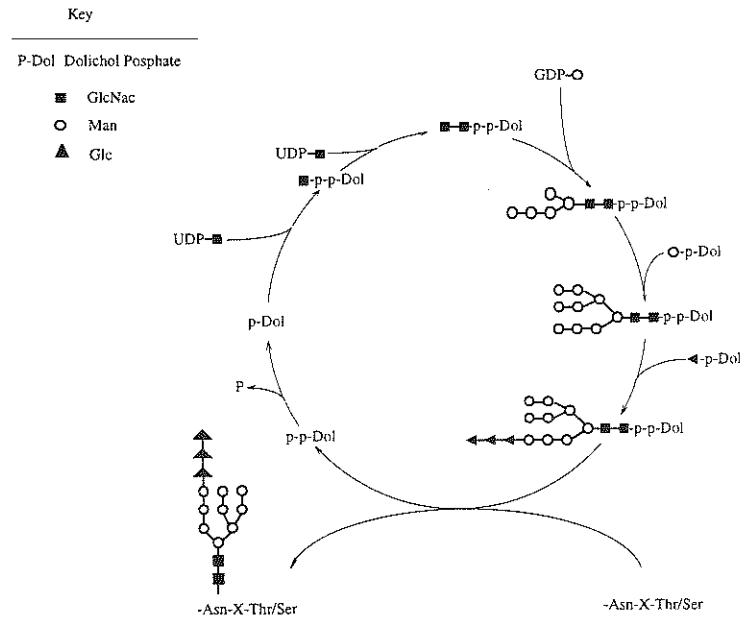


Figure 5. Dolichol Phosphate Cycle: synthesis of the lipid-linked oligosaccharide precursor and its subsequent transfer to the polypeptide.

Transferrin synthesis starts with the association of the mRNA and ribosomes in the cytoplasm. The transferrin polypeptide chain formed initially is nineteen amino acids longer than the final molecule. Before secretion these aminoacids are excised by proteolytic enzymes[99,100]. The shortening of the initial translation product seems to be obligatory because inhibition of this event prevents secretion. This restricted element likely entails the signal moiety necessary for translocation of the mRNA-ribosome-complex from the cytoplasm to the rough endoplasmic reticulum (figure 7). On the other hand, inhibition of glycosylation does not prevent secretion[99].

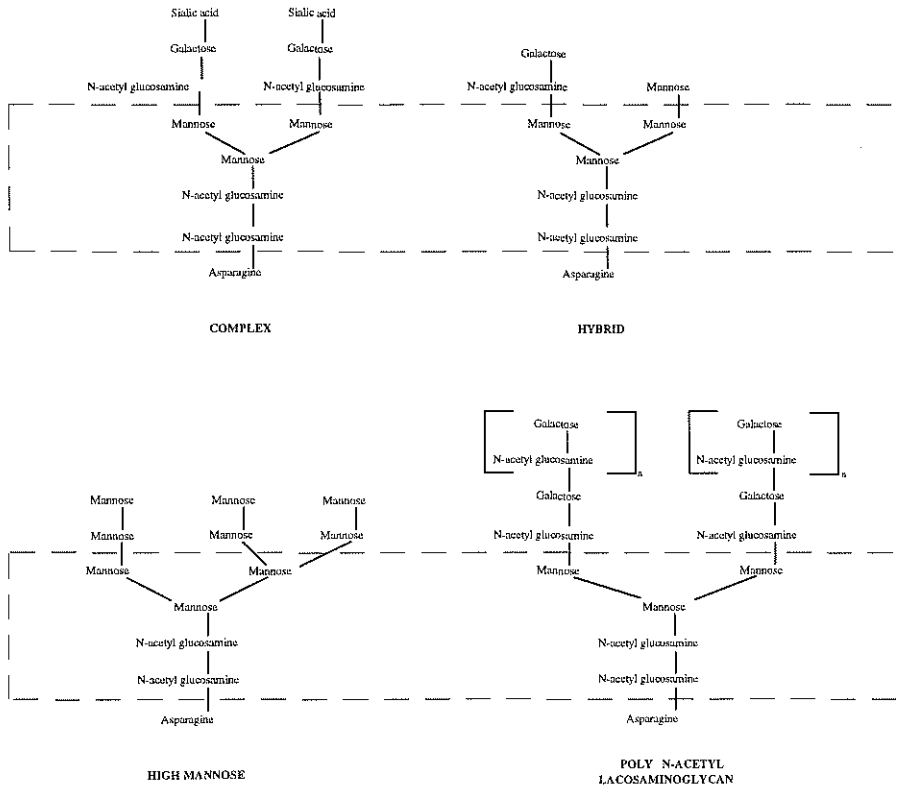


Figure 6. Structure of the four major types of N-linked glycans. The boxed areas enclose the pentasccharide core common to all N-linked glycans. Adapted from reference 96.

N-Glycosylation of proteins is initiated in the rough endoplasmic reticulum (RER) by the synthesis of a lipid intermediate, dolichol-pyrophosphoryl-oligosaccharide, with stepwise addition of sugar residues from activated donor substrates (Figure 5). Next, the complete lipid-linked precursor is tranferred in a single step either to the polypeptide as it is being synthesized and extruded into the lumen of the RER, or after completion of polypeptide synthesis. The membrane associated oligosaccharyltransferase linking the oligosaccharide to the polypeptide is highly specific and transfer will be carried out only to asparagine-residues in the sequence Asn-X-Ser/Thr(Cys), where X may be any aminoacid other than proline or aspartic acid. In general, due to steric constraints only one third of all possible glycosylation

sites are involved in glycopeptide linkage. In the case of human transferrin, two possible glycosylation sites are available and both are utilized.

Up to this point a direct specific coding system is operational, positioning a uniform glycan on specific sites only. The third phase of N-glycan biosynthesis involves the processing and subsequent elongation of the oligosaccharide chains to their final form by the sequential, and in some cases mutually exclusive action of a number of highly specific glucosidases, mannosidases and glycosyl-, phosphoryl- and sulfotransferases as the glycoprotein passes through the different compartments of the Golgi system (Figure 7).

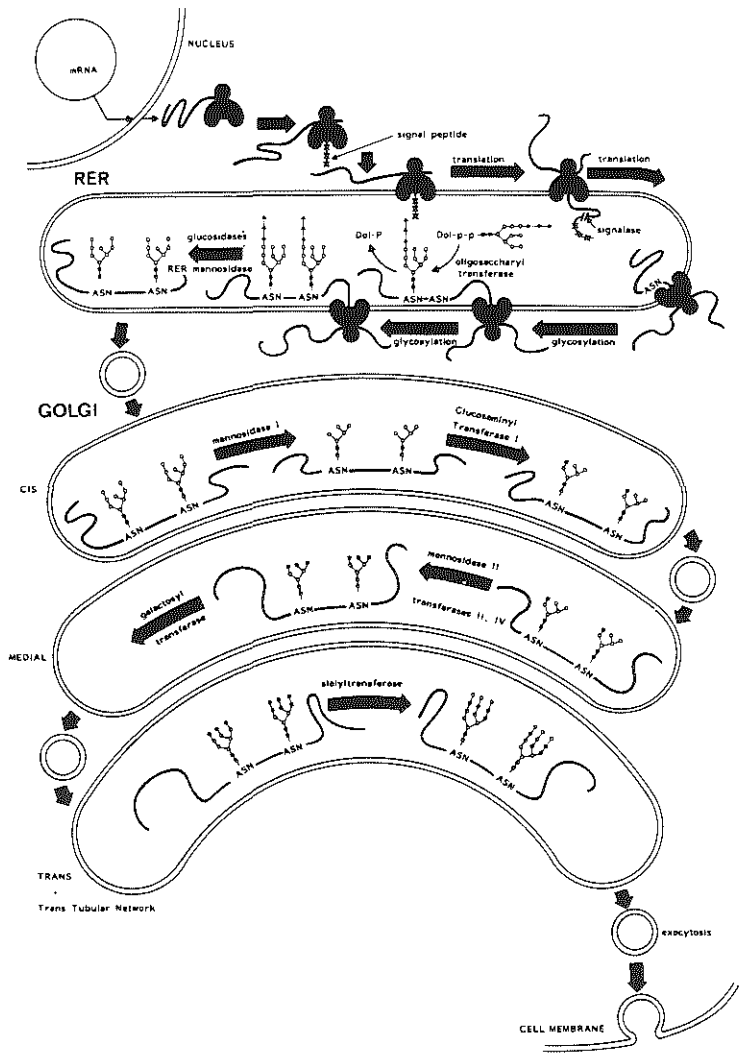


Figure 7. Biosynthesis of glycan chains in the RER and Golgi apparatus. ♦ Sialic acid • mannose, o galactose ■ N-acetyl glucosamine, ▲ glucose.

It is in this last phase that glycan heterogeneity is produced, affecting all of the carbohydrate chain except the core structure containing 3-D-mannose and 2-N-acetyl-D-glucosamine residues common to all N-linked oligosaccharides (Figure 6,7). There is a wide but limited array of possible structures for complex type oligosaccharide chains (Figure 8). A generalisation that can be made is the distinction of four separate classes of N-linked oligosaccharides: the high mannose, complex, hybrid and poly-N-acetyl-lactosamino-glycan types (Figure 6).

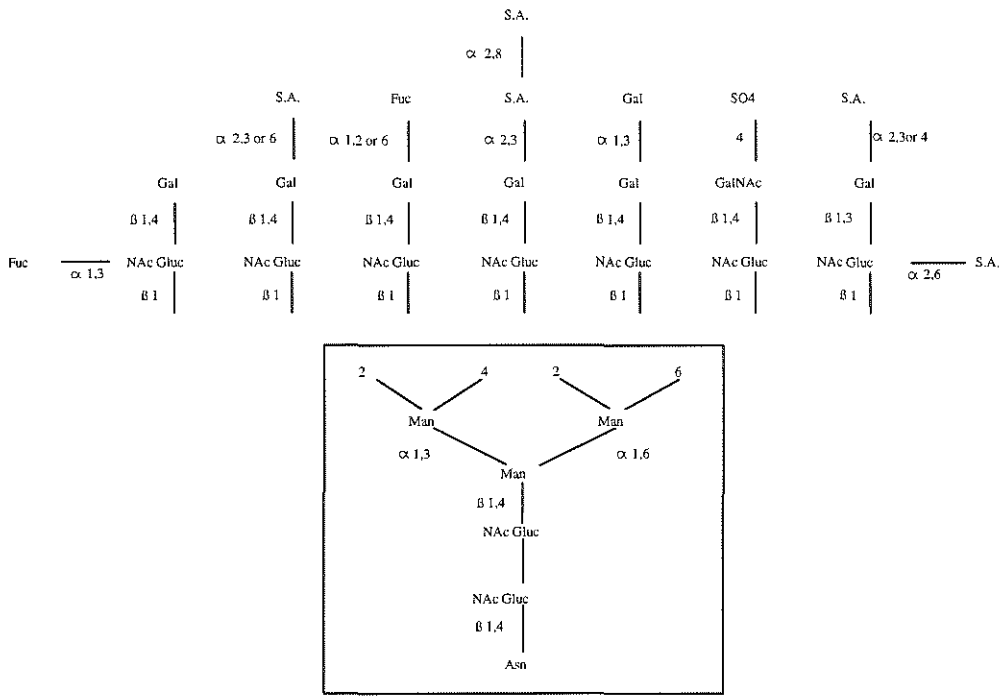


Figure 8. Outer chain sequences found in complex type N-linked glycans. Abbreviations: Man : mannose, NAc Gluc:N-acetyl glucosamine, Gal: galactose, GalNAc: N-acetyl galactosamine, Fuc: fucose, S.A.: sialic acid, SO4: sulphuric acid. The common pentasaccharide core is contained in the boxed area.

Transferrin contains two complex type oligosaccharide chains, differing in degree of branching and terminal carbohydrate composition. This microheterogeneity is due to the fact that glycan synthesis does not take place via an accurate template mechanism, like DNA and polypeptide synthesis. Since instead its synthesis depends on a highly complex and vulnerable orchestration of interactions between glycosyltransferases, glycosidases, polypeptides, differential distribution of enzymes and differential substrate availability, some room for variability is a logical consequence. Illustrating this it can be worked out[94] that while 3 molecules of the same aminoacid can form only one tripeptide, in contrast, taking in account differences in the anomeric configuration and position of the glycosidic bonds, 3 molecules of the same hexose

can form 176 different trisaccharides. Considering the complexity of the synthetic pathways involved, the oligosaccharide structures attached to a particular protein (which generally consist of 11 - 15 components) show surprisingly little variation.
The factors determining which type of glycan a protein will possess at a particular site have not yet been fully determined. The polypeptide moiety itself probably has a strong directional influence. This influence is further modified by changes in the biochemical environment because when glycoprotein genes are expressed in different eukaryotic cells, the processing of starting glycan yields different final structures. A great deal of control is nevertheless exerted on the biosynthetic mechanisms involved in glycan synthesis, since within each different lineage the outcome itself appears to be uniform.
The question still remains whether microheterogeneity is nevertheless a random process with little functional significance. In other words, does microheterogeneity represent "biochemical noise", or does it play a more important role in the organism.

Miscellaneous factors

A number of additional causes of structural variation have been recognised. Like all proteins transferrin is susceptible to nonenzymatic deamidation which will cause anodal displacement. Deamidation is not influenced by the aforementioned causes of heterogeneity with the exception of genetic mutations that lead to changes in the amounts of asparagine or glutamine. The extent to which deamidated transferrins are present in blood is unknown. On close inspection of immobiline gels few very faint extra lines not explained by variation in glycan structure can be seen, but if shown to be related to deamidation this will represent a very small proportion. Nonenzymatic glycosylation of plasma proteins which is most widely known from haemoglobin (HbA_{1c}), but is non discriminative and likewise affects all other circulating proteins and matrix proteins. N-terminal Valines can react with glucose forming a Schiff base resulting in α-amino glycosylation. Additional ε-amino glycosylation of intra-chain Lysines has also been registered in haemoglobin, collagen, low density lipoprotein and serum albumin[101]. Our carbohydrate analyses are consistent with similar nonenzymatic glycosylation in transferrin (table 4), but again this will probably constitute only a very small amount, particularly in view of the in this context relatively short serum half life of transferrin and slow rate of formation of these products. Additional nonenzymatic posttranslational modifications both from endogenous (urea, acetaldehyde, pyridoxal phosphate) and exogenous sources (acetaminophen, aspirin, alkylating agents) have been described by protein chemists. These are not important in this context, but for a review the reader is referred to[101].
At least in chickens, the so called nerve derived transferrin appears to be a separate species of serum transferrin. This transferrin can be distinguished from serum transferrin by monoclonal antibodies[102]. As judged from the source of the monoclonal antibodies used in this study, the structural difference appears to involve an embryonic epitope not expressed in liver-derived transferrin.
Lum et al[103] have isolated a 62000 Dalton transferrin from activated helper-inducer T4-lymphocytes (and not inactivated T4 or other T and B lymphocyte subsets) that constituted more than 60 % of immunoprecipitable transferrin in those cells. Absence of carbohydrates cannot explain the weight difference, therefore other structural determinants are certainly involved. Further structural definition of this substance would be interesting since this transferrin has been implicated in the autocrine pathways involved in control of lymphocyte proliferation.

Chapter 4.

Transferrin functions

Transferrin in iron metabolism

Iron uptake by transferrin

Cellular transferrin and iron uptake

Differential occupation of iron binding sites

Transferrin as Growth factor.

Transferrin in microbiology

Management of physiologic iron toxicity

The discovery of transferrin followed from the work on the bacteriostatic effect of serum. Nevertheless, in many books and scientific articles the function of transferrin is described as restricted to acting as an inert vehicle for the transport of ferric ions through the body, which is an incorrect simplification. Currently the physiological functions of transferrin can be listed as follows:

1. Transport of ferric ions through diverse body fluids.
2. Stimulation of growth and differentiation.
3. Non-immunological defence: bacteriostasis and antineoplastic activity.
4. Protection against toxic effect of metals including iron.

Transferrin in iron metabolism

Early studies on systemic iron metabolism[104] have shown that erythropoiesis is the most important determinant for the rate of plasma iron turnover. Pregnancy can also pose considerable demands on the body, considering that an amount of iron equal to 20-25% of the maternal iron stores is delivered to the growing foetus mainly during the last trimester[105] (figure 9 and figure 20).

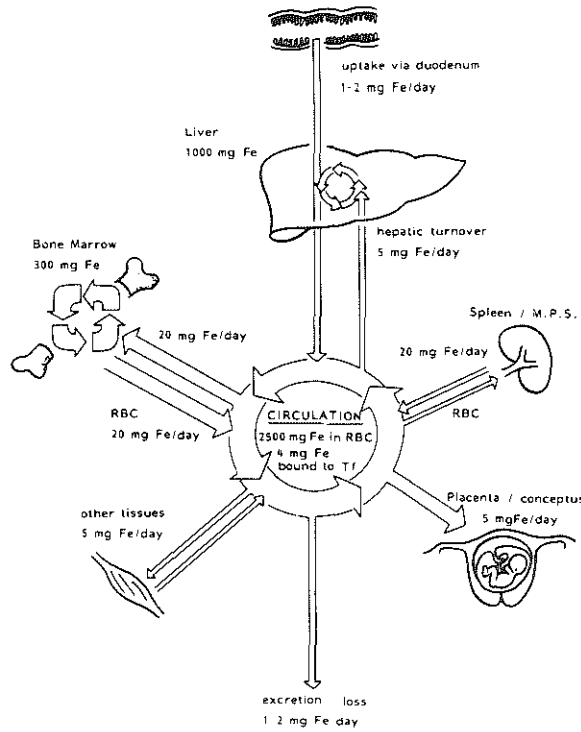


Figure 9. Iron fluxes in man.

The body iron status is regulated at the level of iron uptake rather than at the level of iron excretion. A close relation between the rate of mucosal iron uptake and the rate of erythropoiesis[106] and with the rate of transplacental iron transfer[107-109] has been described. The proposition that transferrin is the only factor connecting intestine and target cells is questionable. Moreover, transferrin is probably not primarily involved in the transport of iron from intestinal cells to the circulation. Iron absorption sufficient to be accompanied by high liver iron stores despite hypotransferrinemia[13,29] suggests that transferrin does not play an essential role in the absorption of iron from the intestine. Using chronic hypoxia as a model to stimulate erythropoiesis in the mouse, Peters and co-workers have shown that the rate of mucosal iron absorption could be dissociated easily from overall erythropoietic rates. Changes in intestinal iron uptake do not necessarily reflect changes in plasma iron turnover. The biochemical mechanism by which the enterocyte responds to various signals remains unknown. The transfer, however, of mucosal iron to the body was shown to depend on bone marrow activity[110,111]. The same conclusion was reached earlier for the coupling of mucosal iron uptake and placental iron transfer in rodents[109,112].

Transferrin does play an important role in the continuous redistribution of iron between the sites of storage and utilisation (figure 9). This again is best illustrated in the rare cases of atransferrinemia. Undoubtedly transferrin plays an important role as an iron carrier effecting transport between liver, bone marrow, spleen and other tissues. In pregnancy iron is transported to the syncytiotrophoblast via transferrin and the transferrin receptor. No alternative routes to the embryo are known in humans. The role of transferrin in brain iron metabolism is less clear.

Transferrin levels are about 200 times lower in cerebrospinal fluid than in serum, but this is matched by the lower rate of iron turnover in the brain. Unfortunately no data are available on transferrin levels in cerebrospinal fluid of atransferrinemic idividuals. Possibly in these individuals at the extrahepatic sites the transferrin gene is translated at for the organ involved normal rates, since trace quantities of transferrin are reported to exist in the serum of these individuals and extrahepatic transferrin synthesis is also probable because absolute atransferrinemia conceivably would lead to such serious disturbances in brain development that the embryonal stage would not likely be survived. A similar reasoning can be pursued for the production of transferrin by Sertoli cells; likewise it is not known whether this system is disturbed in atransferrinemia.

Iron uptake by transferrin

In contrast to the abundance of information on iron uptake from transferrin is the scantiness of information available on the physiological acquisition of iron by transferrin.
In general terms, three systems can be implicated. Firstly, iron could be acquired by transferrin during intestinal uptake. Secondly, uptake could be possible in the process of recycling of the iron released in the physiological turnover of iron-containing enzymes and proteins, especially haemoglobin. This proceeds mainly in the spleen and bone marrow, but also in other cells belonging to the mononuclear phagocyte system such as the Kupffer cells of the liver. Thirdly, iron could be obtained directly from iron stores, principally located in hepatocytes in the form of ferritin and hemosiderin.
A small number of studies aimed at clarifying the iron-release mechanism have been reported. In these, transferrin does not appear to play a direct, active role in release of iron from hepatocytes[113] or macrophages[114]. This confirms expectations based on logical arguments. Iron-free transferrin binds weakly to the transferrin receptor, and therefore the passage of iron from the cells to transferrin is not expected to depend on interaction of the protein with the cell surface receptor. With respect to hepatocytes, the experimental results are difficult to interpret since both iron influx and efflux may occur at the same time. Influx has been estimated at $4.4 \cdot 10^4$ iron atoms / cell / minute, but this must be read with caution as influx in hepatocytes is not saturated with increasing transferrin concentrations and also it increases with lowering oxygen concentrations. Efflux data are not available.
In addition to this, results are complicated by the presence of the asialoglycoprotein receptor system in hepatocytes. Both apotransferrin and iron-containing transferrins can be endocytosed through the asialoglycoprotein receptor, but in my opinion this system does not play a role of quantative importance in normal iron metabolism (tables 11 and 12), or even in iron metabolism in alcoholics.
Studies on iron uptake from the intestinal lumen show that somewhere along the line transferrin is involved in its transport to the tissues. No decisive information, however, is present on the stage at which iron is bound to transferrin. Finch and Huebers postulated a central role for transferrin in mucosal uptake of iron. Transferrin secreted in the gut was postulated to bind intraluminal iron and to donate it to enterocytes. Transferrin receptors have been demonstrated neither on enterocyte microvillous membranes nor on basal membranes. The intraluminal transferrin hypothesis must in my view be discounted. The fact that iron absorption is maximal in the duodenum implies that the actual mechanism is either exquisitely dependent on physicochemical characteristics of iron salts themselves, or the result of specific characteristics of duodenual enterocytes, or a combination of these two elements. What happens at the basal

side of the cell is also still not revealed. Recently, Sibille et al[115] demonstrated that ferritin is released from macrophages after phagocytosis of red blood cells. A considerable portion of the iron in this ferritin appears to be easily dissociated and shifted to transferrin in culture media. In intestinal iron uptake, red blood cell turnover and in transplacental iron transport such a mechanism could in principle be operative. Also, as suggested[115,116] ferritin, besides being a protein of iron storage, could be an important iron transport protein since circulating ferritin is rapidly cleared by the liver and its iron recovered and stored by hepatocytes.

Cellular transferrin and iron uptake

Uptake of iron from transferrin has been very extensively investigated. Two main mechanisms of uptake have been proposed that have found conclusive or at least very strong experimental support:

1. Receptor mediated endocytosis with pH dependent iron release from transferrin.

2. (Reductive) release of iron from transferrin at the cell surface .

Mechanisms such as adsorptive endocytosis of transferrin, fluid phase pinocytosis[117], diffusion[118] and others have been proposed, but lack substantial experimental support in favour of a physiological role in normal adults. A small note in this context: still common to all theories designed to explain iron uptake is that neither of them offer an explanation for the transplasmalemmal transport or the transcytoplasmic transport of iron to its final cellular destinations, but this is not to nullify the achievements in this field described below.
These two mechanisms exist in various cell lines with different quantitative importance in each. While generally accepted to be the mechanism of iron uptake in the red cell lineage and in placenta, receptor mediated endocytosis is disputed to be the quantitatively most important mode of uptake in hepatocytes and neoplastic cells[119,120]. Transferrin concentrations attained in physiological conditions are above those needed to saturate the transferrin receptors. Under those conditions isolated rat hepatocytes take up iron from transferrin predominantly located at the plasma membrane, receptor mediated endocytosis therefore playing a less important role in this cell type with respect to iron uptake.
Conjecturing on the available knowledge of the diverse mechanisms as described below, reductive release at the cell surface probably qualifies as the phylogenetically oldest method of iron uptake. A homologous mechanism has been demonstrated in unicellular eucaryotic organisms[121]: through the combined action of a plasma membrane ferrireductase and an Fe(II) transporter iron uptake is effectuated and regulated in response to environmental demands in yeast. Receptor mediated endocytosis developed in multicellular organisms where specialisation of tissues requires high efficiency and high speed of uptake. Bone marrow and placenta are such examples; the rate of uptake in nucleated bone marrow cells for instance is estimated at 800,000 iron atoms per cell per minute, while in liver cells this figure is about 40,000 iron atoms per cell per minute, and substantially lower figures are found for resting cells such as fibroblasts.

The receptor mediated endocytotic cycle

The endocytotic recycling pathway is known from *in vitro* studies of iron absorption by cell cultures. After having been described by Morgan and Appleton in rabbit reticulocytes[122], receptor-mediated endocytosis has been studied in a large diversity of cell types. For instance, HeLa cells[123], isolated mouse hepatocytes[124], isolated rat hepatocytes[117], HepG2 cell[87] and K562 cells[125] have been studied.
A description of the receptor mediated endocytosis of transferrin is given in Figure 10.

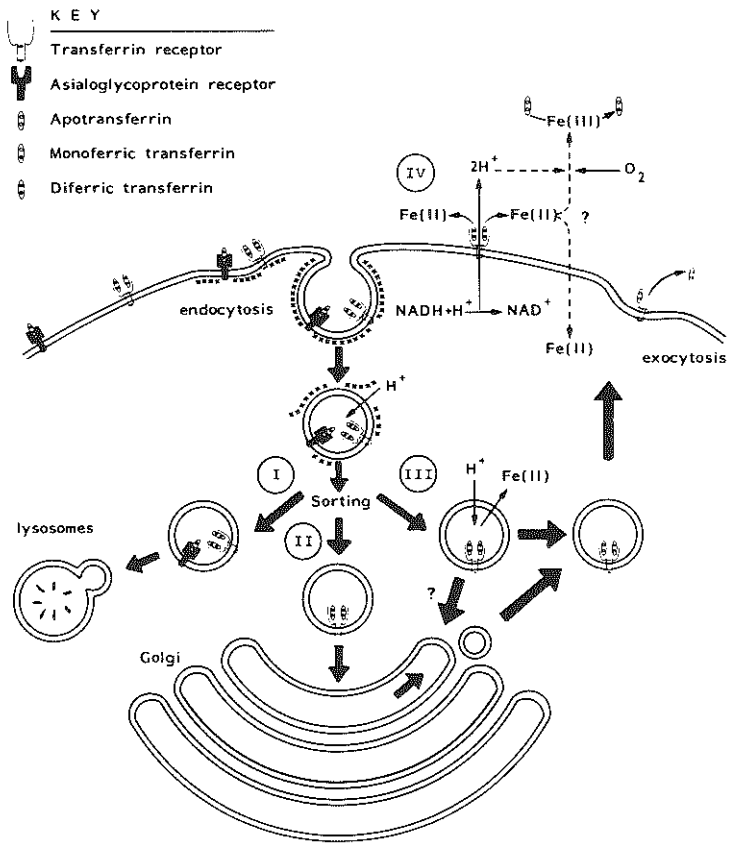


Figure 10. Processes involved in the intracellular transport of iron. Transferrin will bind to a transferrin- or alternatively to an asialoglycoprotein receptor on areas of the cell surface specialised for uptake by endocytosis. The iron release from vesicles requires acidification of the endosomes. An intracellular sorting mechanism directs the iron-triansferrin-receptor complex to proper destinations (I, II and III). After release of iron the transferrin-receptor complex is either externalised undegraded (III), or degraded in lysosomes (I). Route II is involves resialylation of transferrin and/or its receptor and subsequent recycling to the cell surface. In route IV iron bound to transferrin activates a transplasmalemmal NADH-oxidase, possibly with the concomitant reductive release of iron (II) from transferrin and uptake of iron

Cell surface transferrin receptor binds diferric transferrin from the incubation medium, with progressively lower affinities for monoferric and apotransferrin [244]. As reported in the introduction, the K_a for the binding of 2FeTf is 20-fold higher than that of apo-Tf, whereas the two monoferric forms bind with intermediate affinity. This is in agreement with earlier studies *in vitro* as well as *in vivo* with respect to the iron donating behaviour of both monoferric transferrin species[81-83]. Either with or without transferrin bound to it, within seconds after being cycled to the cell surface transferrin receptors are concentrated in dimples in the cell surface coated on the cytoplasmic side by an electrondense bristle-like sheet, hence named coated pits[126,127]. This sheet was later shown to be composed of clathrin molecules, responsible for automated formation of vesicles. At these coated pits receptor molecules randomly (re-) inserted in the plasma membrane are trapped, irrespective of the identity of their ligand, and through this clustering of receptors to specialized fragments of the cell surface and consequent concentration of ligand molecules substantial enhancement of the efficiency of the connected uptake system is achieved.
Through further invagination of the cell membrane this dimple in the cell surface evolves into a rounded intracellular vesicle, initially connected to the surface by a narrow neck (often tortuous and can be up to 1000 nm long). After this the clathrin-molecules segragate from its cytoplasmic face to return to the cell surface to initiate a new cycle, and the neck is lost. Common terms for these vesicles are endosomes or receptosomes (from each coated pit a receptosome is formed every 20 seconds; a typical cultured cell is estimated to form up to 3000 vesicles per minute). At that point the vesicle size has increased through uptake from the cytoplasm to a size of about 200 nm. Size also increases as a result of fusion of receptosomes.
Receptosomes have been shown to move through the cytoplasm. The typical saltatory movement and the fact that it can be arrested by colcemid provide evidence that they move along the intracellular tracks laid out by microtubules. Another important property of receptosomes is the fact that they become acidic. Depending on cell type a pH varying from 6.4[128] to 5.5 is reported[125,129], but generally it is accepted that in receptosomes involved in iron uptake a pH of 5.5 is attained. Receptosomes move towards elements of the Trans Reticular Golgi apparatus and fuse with these elements. After approximately four minutes on the surface[125,130,131] the receptor appears clustered in vesicles and in tubulovesicular structures near the plasma membrane[128,132-135]. At this stage, soon after internalisation, the transferrin receptor-ligand complex is segregated from other receptor-ligand complexes that have co-migrated into the coated pits.
With respect to iron metabolism, a triple role for the acidic pH in these compartments is thought to exist:

1. Sorting of receptor ligand complexes.

2. Dissociation of iron from receptor bound transferrin.

3. Recycling of transferrin receptor - transferrin complex to the cell surface and subsequent dissociation of apotransferrin.

The first role: Stoorvogel et al. reported[136] that treatment of HepG2 cells with the lysosomotropic agent primaquine does not impede transferrin receptor internalisation, but it

totally disrupts the sorting of asialo-orosomucoid and transferrin and concluded that an acidic environment is a prerequisite for sorting. Turkewitz et al. have provided a chemical basis for this observation[137]. The first effect of pH-lowering is to induce a reversible conformation-transition of the transferrin receptor leading to self-association via the ectodomains[137]. Gradual reduction of pH upon penetration of the interior of the cell is hypothesised to be sufficient to induce clustering behaviour, resulting in differential concentration of membrane proteins. The latter results in sorting of unoccupied and occupied receptors and likewise results in the segregation of other pH-sensitive receptors[138]. Pertinent to this attractive hypothesis is the fact that respective conformational transitions of different receptors and subsequent self association occurs at different pH-levels. Thus, in this scheme the diverse physicochemical properties of the receptor-ligand complexes would allow for the complex process of early endosomal sorting without the specific involvement of cytosolic elements. Additionally, protease insensitivity and shielding of tyrosine residues could be obtained, as has been reported for the asialoglycoprotein receptors[139], a property obviously necessary for receptor recycling to occur. This may not be an important feature for transferrin receptors since receptosomes have not been shown to contain hydrolases. Also ligands that are transported to the lysosomes do so not directly from receptosomes but via separate smaller coated pits of the Trans Reticular Golgi membrane with which the receptosomes fuse first.

The second role of the acidification of early endosomal compartments is to aid in the dissociation of iron from transferrin. Again two mechanisms are involved. Because three protons are released for each iron bound, and one from the ionization of carbonic acid to form bicarbonate, the association constant for the binding of iron depends inversely to the fourth power of hydrogen ion activity[140]. The strength of binding of iron by transferrin thus falls by orders of magnitude upon lowering of the pH to 5.5. The measured rate of spontaneous dissociation from isolated transferrin in solution however despite this still takes hours for completion and thus fails to account for the observed rate of iron release *in vivo*. An additional mechanism has therefore long been sought and recently been described simultaneously by two groups[141,142]. Apart from binding the transferrin molecule in the pH range achieved in endosomes the receptor appears to facilitate iron release through a pH dependent conformational change in the molecule. Diferric transferrin in solution will release about 18 % of its iron in 3 minutes at pH 5.6. Diferric transferrin bound to its receptor at pH 5.6 is capable of releasing over 50 % of its iron at room temperature in 3 minutes. This rate is likely to be faster at 37 °C and, in view of general estimates of endocytosis cycle time of 2 - 15 minutes[88,127,130,132,136,143,144] (for transferrin estimated at 2-3 minutes) this can account for physiological rates of iron release.

The resulting apotransferrin-transferrin receptor complex is recycled to the cell membrane. In this phase the *third benefit* of lowered pH is involved: the enhancement of binding of apotransferrin to the transferrin receptor at low pH[145]. This is considered an additional means of deflecting the endocytosed transferrin from lysosomal degradation and is certainly essential for the quick return of processed transferrin to the cell surface. Upon its return to the cell surface and subsequent exposure to neutral pH the apotransferrin is rapidly displaced by iron containing transferrins to engage in another endocytotic cycle[87,88,146]. This cycle has been measured to repeat 300 times before degradation of the transferrin receptor[147]. Current theories on the endocytosis pathway hold that the destination of a receptor and ligand is ultimately decided in the Trans Reticular Golgi where small (80 nm) coated pits have been shown to exist that concentrate ligands destined for lysosomes. Transferrin and its receptor are capable of bypassing these pits and finally make their way into narrow elongated membranous elements

(like receptosomes intimately associated with microtubules) extending from the Golgi to the cytoplasmic region near the plasma membrane. By fusion with the outer cell membrane transferrin bound to its receptor is exocytosed.
Quantitatively important or not, most cells do take up iron by means of receptor-mediated endocytosis. A close coupling of total transferrin binding and iron uptake has been described for erythroid cells in their various stages of maturation[143,148-150]. The role of factors other than total receptor number that might regulate the rate of iron uptake, such as the rate of transferrin endo- and exocytosis and the efficiency of iron release inside the cell is less well defined. The fact that in many types of cultured cells the close relationship between total receptor number and the rate of iron uptake (V_{max}) appears to break down[146,151,152] suggests the existence of other regulatory principles.
Using primary cultures of chick embryo breast muscle, Sorokin et al.[153] demonstrated that the amount of iron taken up at different stages of differentiation is controlled not solely by the difference in receptor numbers, but also by the kinetics of endocytosis, receptor recycling and possibly by intra- and extracellular distribution of receptors. A comparable conclusion has been drawn by Hradilek and Neuwirt[154] from their study of iron uptake by induced- and non-induced Friend erythroleukemic cells. The V_{max} of iron uptake was shown to increase by 100% after five days induction with dimethylsulfoxide. This increase was not accompanied by a proportional increase in the number of transferrin receptors. In the induced cells transferrin endocytosis and release proceeded at identical rates. Transferrin release in the uninduced cells was slower and a substantial part of internalised radioiron was released. The data suggest differential efficiency of iron release from internalised transferrin and changes in cellular transferrin kinetics to be additional factors involved in determining the rate of iron uptake in developing erythroid cells.

Iron uptake from transferrin located at the cell membrane.

Iron release at the cell surface has definitely been established as the second important mode of uptake[119,124]. The major part of iron accumulated by the isolated hepatocyte can not be accounted for by receptor-mediated endocytosis of transferrin. A number of features of iron uptake by hepatocytes are not compatible with the receptor mediated endocytosis. Hepatocyte iron uptake does not saturate with increasing transferrin concentration, is increased by low oxygen concentration, is not significantly inhibited by weak bases or ionophores and can be inhibited by strong Fe(II) chelators impermeable to cell membranes. Clearly distinguishable from the receptor mediated endocytosis pathway, the mechanisms involved have nevertheless remained obscure.
A new element has been added to this discussion by two groups that have demonstrated a transferrin receptor-associated transplasmalemma NADH oxidase activity in both isolated rat hepatocytes and HeLa cells[155-157]. In the presence of strong cell impermeable Fe(II) chelators such as bathophenantroline or ferrozine, extracellular binding of diferric transferrin to transferrin receptor of rat hepatocytes is followed by reduction of ferric ions to the ferrous state at the cost of intracellular conversion of NADH to NAD^+. This is accompanied by transmembranous electron fluxes and concomitant transport of protons via a Na^+/ H^+ antiporter. The Fe(II) released from transferrin in this reaction is envisioned under physiological circumstances to be transported across the plasma membrane to its intracellular destinations (Figure 10).

Again an important, dual role has been ascribed to the proton flux. Firstly it would aid in the labilization of the ferric iron and secondly it would reduce the redox potential of the Fe(III) bound to the transferrin. The latter is essential in order not to exceed the thermodynamic driving force as provided by the NADH oxidation. On theoretical grounds it has been argued that the overall equilibrium constant in the experimental set up is shifted towards the formation of the iron chelator complex due to the unphysiologically strong chelating effect of bathophenantroline, so that transfer of iron from diferric transferrin can not be taken as evidence of reductive mobilization of iron from transferrin at the cell surface[158].
Although in their calculation the authors used an E_0 value of -0.528 V while -0.310 V has been reported to be the correct figure[2], the point they make is valid in that physiological concentrations of all substances involved in the overall reaction need be taken into account. The problem then is that it is not known which substance functions as the physiological Fe(II) chelator. Assuming that -0.310 V is the correct value for E_0, and incorporating the fact that free Fe(II) concentrations are naturally kept very low in oxygenated cytosol, an Fe(II)-chelator of considerably less strength would suffice to shift the reaction towards reduction of transferrin bound iron. As the authors indicated themselves, another variable in the equation, i.e. the hydrogen ion, may *in vivo* be more favourable to the overall reaction as the pH close to the hepatocyte's exterior surface is in all probability lower than the pH in the bulk medium due to the constant extrusion of hydrogen ions by the hepatocyte[156].
At this moment the stoichiometry of the NADH reductase-reaction in hepatocytes is still uncertain. For instance, it has not been proven that it is actually the iron which is reduced at the cell surface or even whether it is taken up by the hepatocyte through this mechanism. It can be hypothesised that the iron released is immediately oxidised in the surrounding oxygenated body fluid and captured by circulating (apo- or mono-) transferrin and a redistribution of iron between circulating transferrin molecules. This appears unlikely since one of the functions of the iron binding proteins is the prevention of presence of free ionized iron in aqueous oxygenated solution. In particular in close proximity to the cell surface by catalyzing the chain of reactions leading to lipid peroxidation serious cell damage would ensue. Alternatively, transferrin Fe(II) species could react with ceruloplasmin before liberation of the ferrous iron atoms occurs, thus preventing lipid peroxidation, linking the long known ferroxidase effect of ceruloplasmin[159] to transferrin metabolism. Another possibility is that transferrin in conjunction with its receptor through one of its epitopes could activate this transplasmalemma oxidoreductase, employing an alternative extracellular substrate. Either way the net effect of the reaction would be an alkalisation of the intracellular medium In this view the system acts as a regulator of cell function irrespective of iron donation, a view that provides a biochemical basis to the growth stimulating properties of transferrin (see paragraph on early effects related to growth stimulation in this chapter).
Efforts are currently directed at describing this hitherto cryptic transplasmalemmal diferric transferrin NADH reductase. At this moment it is not established whether the above described reductive release of iron at the liver cell surface truly describes the quantitatively most important mode of iron uptake in the liver. On the other hand, general agreement does exist on the fact that isolated rat liver plasma membranes are capable of reducing ferricyanide and exhibit diferric transferrin stimulated NADH oxidase activity (reference[158], own observations). Interestingly, the distribution of this transplasmalemmal NADH oxidase is tissue specific. In contrast to liver cells Hela cells and melanocytes, the reticulocyte displays little NADH oxidase activity[119]. As the latter is almost exclusively dependent upon receptor mediated endocytosis of transferrin for iron uptake, it is highly unlikely that the NADH oxidase plays a role in

mobilization of the ferric ions from transferrin in endosomes to the cytoplasm.

Additional modes of transferrin and iron uptake

A mode of uptake similar to the transferrin receptor mediated endocytosis is followed when transferrin is taken up through the asialoglycoprotein receptor. Following internalisation, processing is different though. In early endosomal particles, possibly the tubulovesicular network identified as the Trans Reticular Golgi, divergence of transferrin-receptor bound and asialoglycoprotein-receptor bound transferrin occurs[136]. The latter is routed towards lysosomal compartments, resulting in degradation of the transferrin molecule and recycling of the asialogycoprotein receptor to the plasma membrane.
A third mode of intracellular transport of both transferrin and its receptor has been reported[160,161]. In this mode, desialylated transferrins and desialylated transferrin receptors are returned to the cell surface at a much slower rate but are resialylated, suggesting some sort of repair mechanism. It is presently not clear which receptor or mechanism is responsible for this diversion of the transferrin molecule to the deeper regions of the Golgi complex. It may function as a means of restoring functional integrity of these proteins, but plays no role of quantitaive importance in iron uptake.
Foetal liver cells diplay an unusual high rate of iron uptake not comparable to its adult counterpart both in rate and in basic mechanism of uptake. For these cells it has been characterised as a nonsaturable, transferrin concentration dependent, adsorptive endocytosis-mode of uptake[162].

Differential occupation of iron binding sites

As discussed in the introduction, the physicochemical properties of the N- and C-terminal iron binding sites are not equal in terms of iron binding. Under conditions prevailing in the circulation the effective affinity constants of both binding sites amount to 1 and 6 x 10^{22} M^{-1} respectively[42]. Since its first description this difference and its physiological relevance have been a widely acclaimed matter of conjecture and debate.
In 1967, Fletcher and Huehns postulated that the N- and C-sites might play specific roles in internal iron exchange. The C- terminal site was hypothesized to be involved in removal of iron absorbed by the intestine and in delivery of this iron to bone marrow and trophoblast; the N-terminal site was assumed to deliver iron preferentially to the iron storing hepatocytes. In vitro and in vivo the iron donating properties of Fe_NTf and $TfFe_C$ did not differ however[81,82], so it is now generally accepted that this hypothesis cannot be maintained.
The *in vivo* occupancy of N- and C-terminal binding sites is also a matter of debate. There are several reports about the ratio of Fe_NTf over $TfFe_C$. Zak and Aisen[77], and van Eijk et al.[90,163] reported predominance of Fe_NTf over $TfFe_C$ in sera of healthy donors. Other groups presented contradictory data for peripheral plasma[41,80,164] and for supernatants of cultured cells[165]. The non-random distribution of iron among the two binding sites is disputed for both healthy individuals[84] and patients with haematological disorders[85]. Serum taken from different vascular compartments has been compared with respect to the relative amounts of apo-, mono N-, mono C-, and diferric Tf. The patterns describing the relative amounts of these four transferrin subspecies did not differ significantly between the different compartments[80]. Under

physiological conditions the different species of Tf apparently do not serve specific functions in directing the flux of iron. The non-random distribution of Fe_NTf and Fe_CTf has remained unexplained.

A different light on the matter comes from two observations. Firstly and central to the hypothesis, pH dependent, receptor induced switch in binding site cooperativity has recently been described[166].Through this modulation of the kinetics of iron release from transferrin, the transferrin receptor plays a key role in the release of iron from transferrin in endosomes. Diferric transferrin bound to its receptor will at the pH prevailing in endosomes release about 50 % of its iron in the time required for a full cycle of receptor mediated endocytosis to occur. This implies that a considerable proportion of internalised transferrin will return to the cell surface in its monoferric state. Saturating conditions exist for most cells with respect to the concentration of diferric transferrin in relation to the receptor affinity constant. Therefore the monoferric transferrin with the lower affinity for the receptor will be displaced by diferric transferrin soon after the transferrin receptor complex has returned to the cell surface. Why then do we still find diferric transferrins in blood ? Probably because the time for such exchange to occur is short. At an estimated density of 1000 coated pits per cell together occupying 1 % of the cell surface, based on the lateral diffusion rate of the EGF receptor it has been calculated that a recycled receptor will meet another coated pit and engage in a new endocytotic cycle within seconds[167]. Presumably this time is short enough for most monoferric transferrins to escape exchange and upon descending the next coated pit proceed to delivery of the second iron atom. Nevertheless some monoferric transferrins will be exchanged. At this point the receptor induced switch in site lability described by Bali and Aisen is interesting. Apart from opening the binding cleft in the C-terminal domain, conformational changes induced by the receptor also influence the N-terminal lobe to such an extent that in doubly occupied transferrins release from the N-terminal site is slowed. At pH 5.6 the overall effect of binding of diferric transferrin to its receptor is stabilisation of iron in the otherwise reactive N-terminal site and labilisation of iron in the relatively stable C-terminal site. Whereas in circulating transferrin release is fastest from the N-terminal site, transferrin bound to its receptor at pH 5.6 thus preferably releases its iron from the C-terminal site. Thus, monoferric transferrins released from cells will preferably be Fe_NTf and considering that the major share of diferric transferrin is metabolised in the bone marrow this may explain the predominance of Fe_NTf over Fe_CTf in peripheral blood.

A simple test of this proposition involved the isoelectric focussing of bone marrow plasma as most of the transferrin turnover via receptor mediated endocytosis occurs in the erythroid cell lineage. Consistent with the former line of thought an increased amount of Fe_NTf in bone marrow plasma compared to the amount of Fe_NTf in peripheral blood plasma was found by us. Returning to the Fletcher-Huehns hypothesis described above, a contrasting view emerges: the different species of Tf apparently do not serve to direct the flux of iron between organs, but the amounts relative to each other reflect the fluxes of iron to these organs. Fe_NTf is more or less produced locally by bone marrow and other cells actively engaging transferrin in receptor mediated endocytosis. This is probably counterbalanced by iron release from stores and organs involved in the recycling of iron from erythrocyte turnover as this will at physiological pH preferably bind to the site in the C-terminal lobe of transferrin. The physiological benefit of favouring the acid stable site over the acid labile site as a source for cellular iron needs has been questioned by the authors who described the phenomenon of site cooperativity switch. It may be that in effect it preserves the transferrin site that at physiological pH most avidly binds iron, thus chelating iron at sites of uptake and recycling most efficiently.

Transferrin as Growth factor.

Serum has a growth promoting effect on cultured cells. Biochemically, a number of serum constituents have been shown to be involved in this growth stimulus. For instance insulin, insulin-like growth factor, interleukin, platelet derived growth factor, epidermal growth factor and serum transferrin have been shown to be independent, effective growth promoting agents. For all polypeptide growth factors including transferrin, binding to specific plasma membrane receptors is an obligatory step in growth stimulation. This has been shown for transferrin by Trowbridge and Omary[168].
The transferrin receptor is present on the surface of proliferative normal cells, and on benign and malignant neoplastic cells[169]. The terminally differentiated cell has been shown to exhibit little or no significant specific binding sites for transferrin[150,170,171]. This is particularly clear in the erythroid cell lineage where the receptor number increases between the early and intermediate normoblast stages, and subsequently the number decreases and finally disappears at the stage of the mature erythrocyte. Since there is a high correlation between the proliferative state and the level of transferrin receptor expression, the question has arisen whether or not a functional relationship between these two exists and what role can be attributed to transferrin in this system. Traditionally, the growth stimulating effect of transferrin has been ascribed to its function as an iron vehicle. The enzyme ribonucleotide reductase, a key enzyme in DNA synthesis, requires iron as a cofactor, rendering it an iron dependent step in cellular proliferation. However, new data have become available showing that this is too limited a view on the role of transferin in stimulation of cellular proliferation.
In this context it is useful to differentiate between early and late growth promoting effects of transferrin. The neuro- and myotrophic role of transferrin will be dealt with separately, since this involves a continuous stimulation of terminally differentiated cells and, in contrast to the early and late effects, as such is not implicated in support of cell division. It might be termed the anti-senescent effect of transferrin.

Transferrin as a mitogenic agent

The question whether transferrin as such has growth promoting properties has long been a matter of debate. Differentiating early and late effects is rewarding. Based on the reported results one may conclude that membranous and intracellular changes are initiated as an early effect, due to a direct transferrin - transferrin-receptor interaction possibly based on an electrochemical effect on the cell. These changes could be regarded as the minimal requirements necessary for late effects to occur, effects that appear to be basically related to iron supply. In a sense these mechanisms function as a positive feedback loop; a self perpetuating automitogenic stimulus. Therefore additional requirements set by other growth factors or intrinsic factors (for instance defined by neoplastic degeneration) are undoubtedly present when transferrin is acting as a growth stimulating agent. Nevertheless, systems in which these demands are met can provide information on the physiological role of transferrin in growth control. Certainly in its early effects this role goes beyond the level of essential nutrient supplier.

Early effects.

The binding of growth factors to the specific plasma membrane receptors promotes generation of early signals in membrane and cytosol. Within a minute, the mitogenic signal is propagated into the nucleus. A variety of biochemical changes is induced, eventually culminating in mitogenesis. Addition of growth factors (or serum) to responsive cells causes changes within a few minutes in the activity of a variety of electrolyte and non-electrolyte transport systems, in intracellular alkalisation, and in membrane ruffling and cell shape[172-177]. For a review see Rozengurth[178].

The rearrangement of transferrin receptors (TfR), resulting in increased TfR numbers at the cell surface at the cost of the intracellular TfR pool, has also been recognised as an early growth factor-induced event[179,180]. For instance, addition of either insulin, insulin-like growth factor, interleukin, or platelet derived growth factor to HeLa cells kept in serum-free medium, induced a rapid ($t_{1/2}$ = 3 min) and concentration-dependent increase in surface TfR number. This response was calcium-dependent but unaffected by the presence of Tf, demonstrating that at least in HeLa cells TfR distribution is ligand-independent[181]. The influence of growth factors on TfR distribution cannot be demonstrated in all cell types. Thus Ward and Kaplan speculated that only cells that have a substantial quantity of TfR's in intracellular pools may respond to mitogen stimulation with redistribution of TfR 's along with other proteins.

The physiological relevance of this early serum-dependent rearrangement of TfR 's is not clear. Iron being required for the activity of ribonucleotide reductase, a key enzyme in DNA synthesis[182], this would appear to be an early target in stimulating cell division. The initiation of DNA synthesis is, however, a late event, occurring 10 to 15 hours after addition of the mitogens. Therefore the early enhancement of the cellular capacity for transferrin-mediated iron uptake can hardly be causally connected with DNA synthesis.

The hypothesis proposed by Sun et al.[183], explaining the growth promoting effect of transferrin should be considered in relation to this discrepancy. Insulin-induced increased exposure of transferrin receptors at the cell surface is thought to cause an increase in the activity of a plasma membrane and transferrin receptor associated electron transfer system; the NADH-diferric transferrin reductase. Reduction of transferrin bound iron is accompanied by proton release with an $H^+/_{e^-}$ ratio of over 50. This has been demonstrated for Hela cells[183] and rat hepatocytes[119]. H^+ generated in this reaction by oxidation of NADH and either simultaneous reduction of ferric ions presented by transferrin (or, as discussed in the section on reductive release of iron from transferrin, another cryptic substrate) has been shown to exchange with external Na^+ through tightly coupled Na^+/H^+ antiporters. Stimulation of this transmembrane redox system is therefore postulated to result in alkalinization of the cytoplasm[183]. On the other hand alkalinization is related to mitogenic action of growth factors[172-173], which thus ties diferric transferrin to early growth promotion. Since not all cells respond similarly to mitogenic stimulation with respect to transferrin receptor redistribution, this early rearrangement cannot be considered as a necessary event in mitogenesis. In principle and at the least, the chain of reactions as proposed by Sun et al. could potentiate the growth- promoting stimulus of the mitogen in special cell types. Furthermore it allows for the lack of receptor redistribution accompanying an early growth stimulating effect of transferrin in some cell types.

Besides intracellular alkalinization and mobilisation of calcium ions, changes such as turnover of phosphatidyl cholines, alterations in the ratios of cyclic nucleotides or redox state of

nucleotides, as well as maintaining redox components in the plasma membrane in the oxidised state[184,185] have been implicated in the control of growth. All of these effects can be directly or indirectly related to the existence of external oxidants acting upon a transmembranous redox system. Artificial electron acceptors, ferric or non ferric type, should be capable of stimulating growth via the transplasma membrane redox system. Studies by Ellem and Kay[185] show that external ferricyanide can stimulate growth of melanoma cells. Ferric sulphate stimulates growth of 3T3 cells[186] and impermeable indigosulphonates stimulate cell division in sea urchin oocytes[187]. Non-ferric artificial electron acceptors such as hexamine ruthenium III and indigotetrasulphonate stimulate growth in Hela cells[188,189]. In fact it has been claimed that all external oxidants with redox potentials above -125 mV can stimulate growth[188]. That modulation of this activity operates as an important principle in development and differentiation has been recognized and elaborated in several zoological investigations. The development of the vegetative pole of sea urchin oocytes can be stimulated by lithium salts at the cost of the development of the animal pole. The reverse can be established with isothiocyanate. Similar antagonistic actions have been shown operational in the development of eyes and brain in squids and in the regional differentiation of neuroectoderm of vertebrates[190].
In this view, transferrin is not indispensable in culture media, but is simply one of the physiological substrates in an indispensable cellular mechanism.

Late effects.

As mentioned, transferrin stimulates cell growth and division by binding to specific proliferation-related surface receptors[168]. For this reason, the pathophysiological behaviour of these specific transferrin receptors must be considered simultaneously.
Generally, proliferative cells in the early S-phase have increased TfR numbers, whereas the TfR in silent cells is nearly absent. A number of exceptions to this empirical rule is known. One of them is the villous cytotrophoblast. This particular cell type has been shown to be proliferative in early pregnancy and to a lesser extent in the term placenta, despite the absence of surface transferrin receptors in situ[191-193]. After fusion and / or differentiation to syncytiotrophoblast the transferrin receptor (again) comes to full expression, a process which can be studied in *in vitro* cell culture systems[256]. The presence of the transferrin receptor on the terminally differentiated syncytiotrophoblast reflects the transfer function of this maternal-foetal barrier layer. Another exception is formed by the terminally differentiated myotube *in vitro*. This cell type also continues to express a high number of transferrin receptors per unit DNA[153]. This may reflect a continued iron requirement, as suggested by studies of Ozawa and Higawaza[194]. They demonstrated that myotubes require more iron than presumptive myoblasts probably because of myoglobin production. A third example is the mammary gland during gland development. The work of Schulman et al.[195] demonstrated that growth of the normal -murine- mammary gland occurs mainly in mammary epithelial cells and is associated with a clear increase in TfR's. As shown by Drewinko et al.[196] for cultured human colon cancer cells, also malignant cells do not necessarily downregulate their TfR number when quiescent. Apparently the expression of the TfR does not only depend on and reflect the proliferative state, but also depends on the cell's specific developmental pathway.
TfR expression is critical for the G_1-S phase transition in malignant as well as in normal T-cells[197]. In tissue cultures of both normal and malignant cells it has frequently been demonstrated that in the early S-phase both the rate of DNA-synthesis and TfR expression at the cell surface as well as in the whole cell are high as compared to the confluent state of the

cell culture[198-200]. In T-lymphocytes, transferrin receptor expression is tightly coupled to prior expression of the specific growth factor interleukin-2. In the normal resting T-cell the transferrin receptor gene is transcriptionally virtually silent, but is activated by mitogenic agents[201]. This response to growth factor stimulation must be distinguished from the before-mentioned early effect; the redistribution of pre-existing TfR's in favour of the cell surface.
There is little doubt that the enhanced expression of the TfR is related to the late growth-promoting effect of transferrin. The question remains whether transferrin as such, or the iron bound to transferrin, is the active agent. In rat thyroid follicular cells (FRTL5 cells)the growth promoting effect of transferrin, as measured by ^{3}H-thymidine incorporation, was mainly confined to cAMP- dependent mitogenic pathways activated by thyroid stimulating hormone and dibutyryl cAMP[200]. Transferrin had no apparent effect on cAMP-independent stimulation of DNA synthesis induced by insulin and IGF-1. Desferrioxamine, a potent intracellular iron chelator, could easily abolish the stimulatory effect of the cAMP-dependent mitogens, suggesting that in this reaction chain iron was the effective agent, and not transferrin or transferrin-bound iron. Stimulation by the cAMP-independent mitogens was much more resistant to desferrioxamine treatment[200]. How exactly iron, transferrin, cAMP, ribonucleotide reductase and transferrin receptor synthesis are causally connected is unknown.
The conclusion that iron is the effective stimulatory agent is strengthened by other observations. Cell lines like K-562, HL 60 and KG-1 have been grown for many years in transferrin-free media containing ferrosulphate[202]. Other evidence comes from studies with the monoclonal antibody 42/6 raised against the transferrin receptor, which in contrast to the monoclonal antibodies OKT9 and B3/25 inhibits transferrin binding in a competitive manner. Growth of KG-1, K 562, SV-cells and transformed 3T3-cells was inhibited by monoclonal 42/6, and addition of soluble ferric nitriloacetate reversed this inhibition[203]. However, these observations cannot be generalised. Growth of CCRF-CEM cells was not re-established with iron supplementation[203]. The same group reported earlier that certain monoclonal antibodies against the transferrin receptor inhibit growth of granulocyte-macrophage-colony forming unit-cells, and that this inhibition could not be reversed by iron[204]. A comparable observation was made by Trowbridge and Lopes[205], with a tumour cell line in which the inhibitory effect of the monoclonal 42/6 on cell growth could not be overcome by iron salts. Moreover the antibodies 42/6, B3/25, and 43/3 inhibit myeloid cell growth whereas they stimulate iron uptake[205,206]. One must realise, however, that in these experiments total effects are in fact measured. Whether proliferation did not occur due to the lack of the so called early effects, or due to the absence of late effects has not been substantiated.

Neuro- and myotrophic activity

It is a long-standing clinical observation that denervation of voluntary muscles inevitably leads to atrophy of the muscles involved. Passive exercise, frequent direct electrical stimulation of muscles and other physiotherapeutic measures have no positive effect on the atrophy. The changes induced are reversible though, as evidenced by the gradual recovery after renervation of these muscles. Thus a nerve-derived myotrophic factor, excreted and transferred to the muscle at the motor end plates, has been postulated.
Initially, embryo extract was shown to be capable of supporting myogenic development in tissue culture. Various other preparations were shown to contain a factor that is capable of replacing embryo extract. Active components were isolated and given several names such as

Sciatin and nerve derived myotrophic factor. The active substance in these various preparations has now been shown to be identical to transferrin[194,207-209]. Not only is the presence of transferrin essential for myotube development, it has also been shown to be essential for the maintenance of the differentiated state of striated muscle tissue by the nerve[210]. The fact that the terminally-differentiated myotube *in vitro* continues to express transferrin receptors[153] may not only reflect a continued iron requirement[194], but also a developmentally regulated condition necessary for the myotrophic factor to be effective.

It is not yet clear whether Tf required for myogenesis and for the maintenance of the differentiated state of muscle[210] arrives from the nerve itself[50,210,211]. Ample amounts of transferrin could be provided from the circulation, suggesting the possibility that the transferrin required is directly routed to its final destination on the cell surface. Again two functions of transferrin must be distinguished here; its role in iron donation necessary for myoglobin turnover and the myotrophic role. In addition to this, the nerve-derived transferrin has been shown to be structurally different from the circulating transferrins[102]. Therefore it is unlikely that circulating transferrin is directly involved. On the basis of currently available data it is most likely that a specific transferrin is produced locally either by the motor neurone itself or by the Schwann cells. In the latter case, transferrin could be donated to the efferent fibre and routed to the final destination. A homologous situation seems to exist in the central nervous system where the oligodendrocytes provide the cytoarchitectual support and define the biochemical microenvironment of neurocytes. Oligodendrocytes have been reported to synthesise and store transferrin[211]. Oligodendrocytes of newborn rat brain have been shown to be transferrin-independent in culture[21] and can even be selected for in cell culture by eliminating transferrin from the incubation medium. Thus oligodendrocytes could possibly deliver Tf to developing neurones and astrocytes. Suggestive in this context are the observations of Hill and co-workers[212] that iron- accumulating areas of the brain are efferent to areas of high transferrin receptor density, at least implying neuronal iron transport. Based on these observations and the apparently developmentally regulated dependence of post-mitotic neuroblasts on transferrin in culture[213] a neurotrophic role has been ascribed to transferrin. It has been reported that nerve derived transferrin is slightly different from the serum protein[102] in all probability due to posttranslational modification, and elucidation of the structure of this particular transferrin will be of interest.

Transferrin in microbiology

Early descriptions of cases of so-called atransferrinemia have pointed at a role for transferrin in antimicrobial defence[13,29]. Besides severe hypochromic anaemia, repeated infections by predominantly Gram-negative bacteria dominate the clinical picture. Furthermore, transferrin appears to be beneficial in gramnegative sepsis[214]. Another member of the transferrin group, lactoferrin, also displays antimicrobial activity against Gram-negative bacteria, but the mechanism of action has not been defined. Initially this antimicrobial effect of (iron-free) transferrin was explained by the competition of transferrin and bacterial siderophores for iron, thus depriving bacteria, but also fungi and protozoa, from this essential nutrient. As an element of the acute phase response the concomitant increase in concentration of lactoferrin at sites of inflammation further enhances the production of an iron-deficient environment that limits bacterial growth.

Endotoxin-binding capacity of human transferrin and Gc-globulin has been demonstrated in a qualitative manner[215]. This binding of endotoxins occurs under more physiological conditions

than described for the high density lipoproteins, to which thus far the role of physiological endotoxin-scavengers had been ascribed. The pyrogenicity and mitogenicity of a number of different endotoxins are inversely related to their transferrin binding capacities[216]. Binding of a wide range of bacterial endotoxins has also been described for lactoferrin. In both lactoferrin and transferrin this effect is blocked by saturation with iron. This and observations on the effect of synthetic chelators on bacterial cell wall stability have lead to an additional explanation of the bacteriostatic potency of both lactoferrin and transferrin. The presence of divalent cations within the bacterial membrane appears to be critical in stabilising the strong negative charges of the core oligosaccharide chain of the lipopolysaccharides molecules. Binding these membrane-associated cations, EDTA releases lipopolysaccharides molecules from the outer leaflet and, upon replacement by nonpolar phospholipids from the inner leaflet, it sensitises the bacteria to the effects of complement and lyzozyme. In a nonphysiological sense antibacterial defence is augmented, as the membrane becomes more permeable to hydrophobic antibiotic agents that are normally excluded by the hydrophylic lipopolysaccharides-barrier. It is hypothesised that, in a manner comparable to the synthetic chelator, lactoferrin and transferrin cause lipopolysaccharides release from Gram-negative bacteria and alter the permeability and sensitivity of the Gram-negative outer membrane.

In some Gram-negative bacteria transferrin and lactoferrin fulfil ambivalent roles. *Neisseria meningitidis* and *Neisseria gonorrhoea,* being Gram-negative organisms, are susceptible to the above described attack on membrane integrity. Competition for circulating iron is not operative in this case, since neither of these bacteria produces siderophores[217], but host transferrin and lactoferrin are utilized for iron uptake.

Meningococci have been shown to possess a specific receptor for human transferrin with a relatively low K_d of 0.7 μm[218]. The relatively low Kd is sufficient for binding transferrin, since in many body fluids transferrin is present in concentrations exceeding 1 μM. Moreover, apotransferrin and saturated Tf compete for the receptor equally well. These two characteristics are not surprising when viewed in the context of the fact that, unlike the situation in eucaryotic cells, Tf is not internalised after binding to pathogenic Neisseria species. Therefore high affinity binding of Tf in favour of apoTf as a means of optimising iron uptake is unnecessary. Low affinity binding results in high turnover of Tf at the cell surface. This may allow the meningococcus to sort the available Tf and remove Fe from iron saturated Tf molecules. The gonococcus also possesses lactoferrin and transferrin receptors that are highly specific for the human proteins[219]. The fact that both menigococci and gonococci do not produce siderophores and express lactoferrin and transferrin receptors, suggests that this property may be at least partially responsible for conferring the human host specificity of *N. gonnorhoea* and *N. meningitidis*.

Another example of an organism that has circumvented this non-specific defence mechanism by incorporation of it in its own iron metabolism is the malaria parasite. Like all cells the malaria parasite is dependent upon an adequate iron supply for its development. In the human host, in its proliferative stage the parasite is present within the red cell lineage. Paradoxically, in this potentially iron-rich environment *Plasmodium falciparum* apparently utilises none of the twenty millimolar intraerythrocytic heme for its iron supply although this intra- erythrocytic parasite possesses proteases capable of degrading the protein part of haemoglobin. Rather the metal is obtained from extra-erythrocytic transferrin[220]. The fact that mature red blood cells have no transferrin receptors seems contradictory. However, evidence has been provided indicating that this parasite synthesises its own transferrin receptors that are subsequently incorporated into the red cell membrane enabling it to take up iron from transferrin[221,222].

Possibly this ability may be a distinguishing feature explaining the fact that *Plasmodium ovale* infection is restricted to reticulocytes: These malaria parasites may not be capable of synthesising transferrin receptors. Along the same line the mitigated clinical picture produced by *Plasmodium ovale* compared to the clinical picture of malaria due to infection by *Plasmodium falciparum* may be explained. Severity of the disease is principally related to the percentage of red blood cells infected. Although the amount of reticulocytes is increased due the haemolytic anaemia induced by the infection, the infectious load attained by *Plasmodium falciparum* in mature red blood cells can never be matched by that of *Plasmodium ovale* in reticulocytes alone.
Irrespective of the question whether a particular organism is either non-specifically hampered in its growth, or specifically dependent upon the presence of iron carrying proteins, both lactoferrin and transferrin appear to play a role in the physiological defence against infection. This role is even more pronounced in the hypoferremic environment induced by interleukin I in the acute phase response. Lactoferrin is probably most important in view of its presence in secreta and secretory granules of neutrophil granulocytes. This is further supported by the fact that lactoferrin retains its high affinity for iron at the low pH that can exist at sites of inflammation, whereas transferrin bound iron is labilised at a low pH.

Management of physiologic iron toxicity

Respiring cells are under the continous stress of free radicals. Free ionized iron is therefore a potentially very toxic substance in oxygenated fluids as it will catalyze the formation of hydroxyl radicals, initiating the chain of reactions leading to lipid peroxidation and cell damage[223]. These are constantly being introduced either from exogenous or from endogenous sources as a consequence of normal metabolism. The continuous formation of superoxide anions and hydrogenperoxide exposes cells to the constant risk of being attacked by hydroxyl radicals which can be formed from these two precursors in the presence of transition metals.
Iron bound to transferrin or lactoferrin has been shown not to catalyze the formation of hydroxyl radicals. Therefore, one of the important functions of the iron binding proteins is the prevention of presence of free ionized iron in aqueous oxygenated solution. Inhibition of free radical formation by endogenous free radical scavengers and enzymatic defense systems in conjunction with the binding of free iron by the iron carrying proteins constitute a vital buffer against the unabating oxidative stress of aerobic life.

Chapter 5.

Structural characteristics of transferrin microheterogeneity

Describing microheterogeneity: combination of separation by isoelectric focusing with separation on the basis of degree of branching.

Preparative isoelectric focusing in immobilized pH-gradients.

Carbohydrate analysis of microheterogeneous forms of transferrin

The following 3 chapters cover the experimental work performed. Part of it has already been integrated in the preceding chapters as this would aid in understanding the principles that govern the phenomenon of microheterogeneity, which was after all the starting point of these studies.

The choice between various methods of describing microheterogeneity which can be performed with precision and reproducibility is very limited when looking for practical methods applicable to small quantities of sample. Having reviewed the available techniques isoelectric focusing was deemed the most suitable. In performing the work I have taken advantage of the wide experience in application of electrophoretic techniques (in particular isoelectric focusing) available in our department. Disregarding this practical, local advantage isoelectric focusing is still the most convenient method to describe transferrin microheterogeneity.

Interpretation of the patterns is a related problem: what you see is what you get. Therefore carbohydrate analyses were performed to assess the exactitude of assumptions on the structure of transferrin fractions isolated by isoelectric focusing. For this purification of transferrin subspecies, a protocol for isolation by isoelectric focusing has been developed, and these isolated subspecies have also been used in the various tests aimed at discerning a function of transferrin that might be modulated by variation in the carbohydrate structure, which was a hypothesis developed on the basis of changes observed in the microheterogeneity pattern in a number of (patho-) physiological conditions. An account of results is given in these following chapters. In order to optimize legibility of text and tables, materials and methods and standard

deviations of measurements are described in detail in appendix A and B.

Describing microheterogeneity: combination of separation by isoelectric focusing with separation on the basis of degree of branching.

In the context of this chapter microheterogeneity is defined as the structural variation in monomeric protein due to variation in N-linked glycan structure. Many methods have become available that in one way or another can most often only partially describe this structural diversity. In principle exact description of glycan stucture is possible, but in view of the relatively large amount of protein needed in such analyses, without exception also impractical. Fortunately, transferrin is relatively simple with respect to its glycosylation. Most serum glycoproteins contain 3 or more N-linked glycans and a number of O-linked glycans. Transferrin contains no O-linked glycans and only 2 N-linked complex type glycans, rendering the electrophoretical behaviour relatively simple to describe. By the same token Concanavalin A affinity chromatography will yield a limited number adequately separable fractions of almost unequivocal definition. (figure 11).

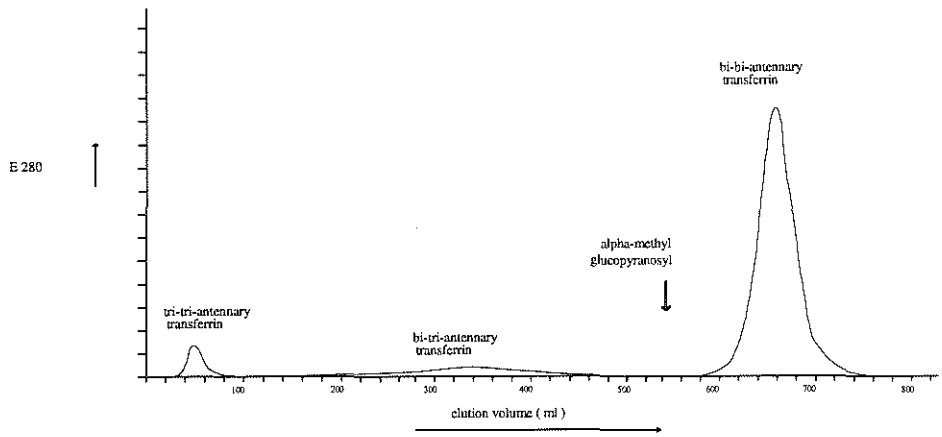


Figure 11. Concanavalin A Sepharose chromatography of human serum transferrin.

Isoelectric focusing in Immobilines is one of the most sensitive methods available to separate molecules on the basis of differences in ionizable groups. It makes good use of the fact that the net electric charge of proteins in aqueous fluids is pH dependent. Immobilines are used to create a fixed pH-gradient in a gel that will itself not be altered by the electric field that is applied to it. Proteins added to the gel however will move between cathode and anode for as long as they have not reached that position in the gel where the pH is equal to its isoelectric point, causing the protein of interest to accumulate at this particular point, or in other words to be focused to that position in the gel.
Previously isoelectric focusing was performed using ampholine-mixtures to create the pH-gradient. In principle this is a similar technique, but because the ampholines are dissolved in the gel rather than fixed to the meshwork of gel fibers, pH gradients are steeper and separation of transferrin subspecies differing 0.1 pH unit was incomplete. The novelty of the more recently

become available Immobiline gels lies in the fixation of the pH-gradient onto the matrix of the gel, which allows for much shallower pH-gradients to be created. Hence, accurate and reproducible quantification of several transferrin relative to each other became feasible (figures 12 & 13).

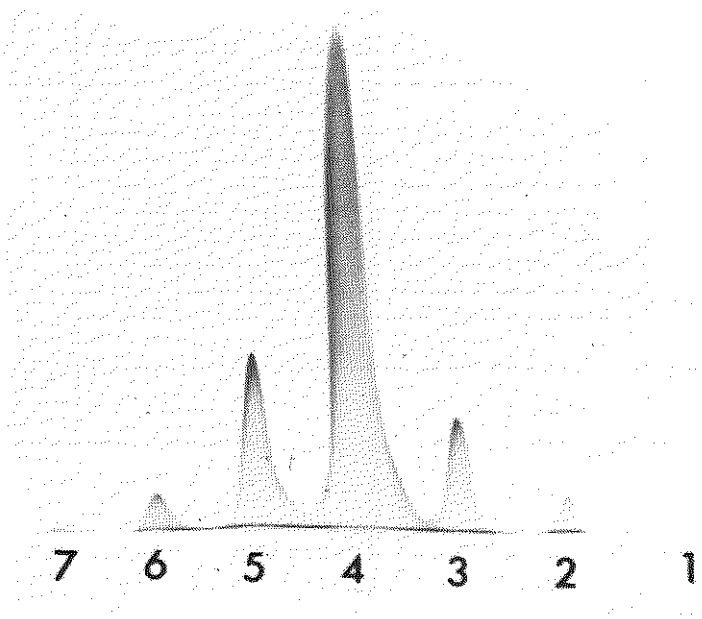


Figure 12. Crossed immuno isoelectric focusing of serum. Numerals indicate the number of sialic acid residues per protein molecule. Cathode at right and bottom for first and second phase, respectively.

Initially quantification was performed by a crossed (rocket)immunoelectrophoresis. The area under the curves was measured and by division on the sumtotal of surface area of all fractions the relative amount of each could be determined. Later after purchasing a laser densitometer we were able to quantify the relative amounts by measuring the absorbance in the gel after immunofixation and subsequent staining for protein. This gives numerically identical results, but is a much less labour-intensive method with even better base line separation and reproducibility of results (figure 13).
Purposely simplifying the picture somewhat (4th paragraph chapter 3) one can safely assume that for diferric transferrin heterogeneity visualised through variation in the isoelectric point is in a single individual the reflection of variation in the number of the sialic acid residues per molecule. As explained in chapter 3, in complex type glycans the degree of branching of the complex type glycans determines this number. Further variation can be present as a result of either aberrant or incomplete synthesis or desialylation. To be able to asses the influence of the latter two factors an additional analysis was performed on the three transferrin fractions that can be separated by degree of branching of glycans by concanavalin A affinity chromatography. The endresult is the distinction of 18 transferrin fractions (table 2).

Table 2. Transferrin microheterogeneity in normal serum as distinguished by degree of branching of glycans and by number of sialic acids per molecule (expressed as fraction in %).

Sialotransferrins	Concanavalin A separation			Total
	bi-bi-antennary	bi-tri-antennary	tri-tri-antennary	
0	-	-	-	-
1	-	-	-	-
2	2.3	0.2	-	2.5
3	7.1	0.2	-	7.3
4	**65.4**	0.8	0.1	66.3
5	0.7	**18.0**	0.2	18.9
6	-	1.1.	**3.7**	4.8
7	-	-	0.1	0.1
8	-	-	-	-
Total	75.5	20.3	4.1	99.9

Trace quantities of the 0, 1 and 8 - sialotransferrins are visible on gels but have been disregarded in this table. In essence the graphs shows that the 4, 5 en 6 sialotransferrins can reliably (87.2 out of 90.1 correct) be interpreted as signifying the amount of transferrins carrying predicted, exemplary complex type glycans. Therefore, by virtue of its comparatively simple carbohydrate structure, we could also use the isoelectric focusing of transferrin as the probe through which changes in protein glycosylation in physiological and pathological states could be monitored.

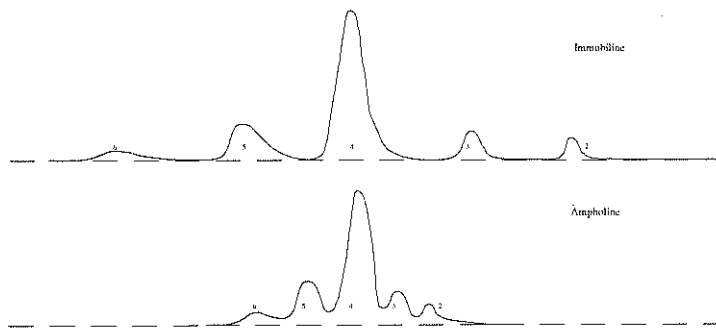


Figure 13. Top half: densitometric scan of sialotransferrins separated on Immobilines. Bottom half: separation in ampholine gel.

Preparative isoelectric focusing in immobilized pH-gradients.

Overloading a gel generally leads to adverse consequences when separating compounds electrophoretically. A gel is essentially a volume of water coordinated by crosslinked, tangled networks of hydrophilic polymers, which second to the water, is the most abundant element of the gel. Small cations and anions are also present in minor amounts. The affinity of the polymers prevents the network from collapsing onto itself, and also prevents the water from flowing away. Upon loading of the gel with the substance of interest, macromolecules spread in the gel, competing for free water molecules. For isoelectric focusing in immobilized pH-gradients in 5% T gels, the maximumloading capacity has been shown to be fixed at 40 - 45 mg/ml gel, irrespective of molecular mass and surface charge of proteins investigated[224]. Relatively few free water molecules are available to proteins that have to gain access to the gel. Focusing enhances this problem; having arrived at its pI-position, focused protein accumulates and increasingly competes for free water molecules. Focusing powers are indeed so strong that concentration of the proteins proceeds to the point where osmotic effects lead to the absorption of water from surrounding gel regions. Movement in the Y-axis being limited by isoelectric forces,and movement in the X-axis simply being limited due to the fixed size of the gel, this osmotic effect leads to expansion in the vertical axis, stretching the gel fibers and cusing local, visible swelling of the gel at the various pI-sites at first.

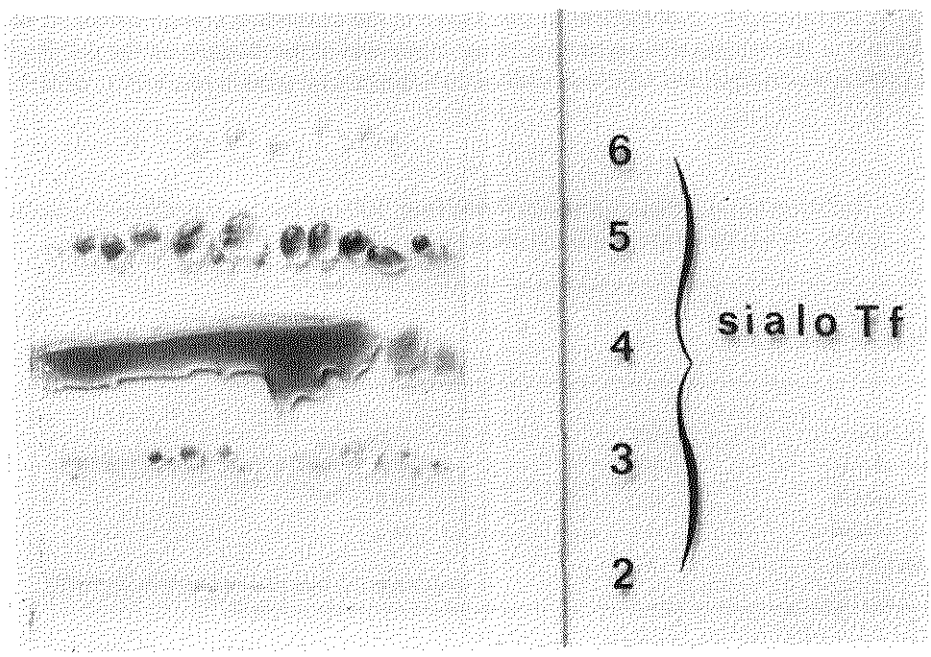


Figure 14. Preparative isoelectric focusing of diferric transferrin in Immobiline gels. Droplets of concentrated microheterogeneous forms of transferrin have formed on the surrface of the gel after prolonged isoelectric focusing.

Sooner or later, depending on the tenacity of the gel, the physical capacity of the gel is overstretched. Liquid accumulation at the pI-sites proceeds to the point where gel affinity forces are defied and fluid escapes from the matrix onto the surface, inevitably containing the focused protein at its maximum prevailing concentration. This effect becomes visible in the formation of droplets on the surface of the gel. Movement of the droplets will not occur, as protein outside its maximal concentration absorbs water from the gel, resulting in loss of the osmotic driving force and reentry in the gel, in turn followed by transport to its pI-position. The size of the droplets is therefore dependent upon the amount of protein loaded onto the gel.
Inevitably, due to the overriding of the buffering capacity of the immobilines at and near to the pI-positions of the proteins to be separated, the separation of proteins is reduced. In the present experimental design the transferrins are separated by distances that tolerate this regional blunting of the pH-gradient caused by local high concentration of protein. Nevertheles if left uncontrolled merging of fractions would occur and for this reason droplet sizes need to be restricted, which can be done by simply removing them before loading additional sample on the gel. Another important feature in our approach has also been that we have started out by isolating blood from transferrin-C_1 donors and thus genetically homogeneous transferrin. Other permissive factors are the fact that transferrin is a stable protein at room temperature, well soluble in aqueous solutions, not sensitive to anodal oxidation or cathodic reduction and resistant to spontaneous and microbial decomposition.
Equilibrating an immobiline gel with protein quantities by far surpassing the loading capacity of the gel while maintaining excellent separation of the subfractions, diferric microheterogeneous forms of transferrin can be isolated easily, which is demonstrated most persuasive in figure 14. Not evident in this figure is the distinctive orange colour of diferric transferrin that is unmistakably present on visual inspection of the droplets on the gel. Recovery of the protein was simple and effective with an 88% direct total protein recovery (table 3).

Table 3. Isolation of microheterogeneous forms from 100 mg human transferrin-C_1 isolated from serum by immunoaffinity chromatography.

Transferrin fraction	pI	mg recovered
2 Sialotransferrin	5.7	2.5
3 Sialotransferrin	5.6	8.2
4 Sialotransferrin	5.45	62.0
5 Sialotransferrin	5.3	14.2
6 Sialotransferrin	5.2	1.5

Results were checked by analytic isoelectric focusing of isolated fractions and showed besides the desired fraction at the most a trace amount (< 2 %) of the first next more acidic transferrin

for each sample.
The transferrins described in table 3 were used for carbohydrate and aminoacid analyses, in the incubation experiments on hepatocytes and in determining the affinity for placental transferrrin receptors. The incubation of human bone marrow, the activity of the NADH reductase and iron binding constants were assessed employing microheterogeneous forms that were defined even more unequivocal as these were separated first on the basis of glycan branching by concanavalin A affinity chromatography (figure 25). This resulted in the isolation of bi-bi-antennary tetrasialotransferrin, bi-tri-antennary pentasialotransferrin and tri-tri-antennary hexasialotransferrin. For example, disturbance of results by tri-tri-antennary tetrasialotransferrin is not to be expected in these experiments.

Carbohydrate analysis of microheterogeneous forms of transferrin

The structure of most of the N-linked glycans of human transferrin has been established by techniques such as methylation analysis and ^{1}H NMR spectroscopy[225,226]. In these cases glycans are either chemically or enzymatically separated from the protein prior to the carbohydrate analysis. Therefore quantification of the relative amounts of the microheterogeneous variants of a glycoprotein defined by these techniques cannot be achieved and also fractions occurring in very small amounts are likely to be lost because of necessary simplification steps in the interpretation of results.
Only indirect evidence for the structural composition of the carbohydrate moiety of microheterogeneous components is available. The objective of these analyses was to check the accuracy of the nomenclature of subfractions, as adopted on the basis of the fact that the microheterogeneity pattern can be reduced to one single fraction (asialotransferrin) in a stepwise fashion by sialidase.
Transferrin isolated by immunoaffinity chromatography of serum obtained from healthy donors, all shown to be homozygous for the C1 allele by analytical isoelectric focusing, was used in these aminoacid and carbohydrate analyses. Subfractions designated 2 sialo to 6-sialo were prepared as described in the paragraphs on preparative isoelectric focusing in immobiline gels.
Aminoacid analysis of these subfractions revealed no differences between the microheterogeneous forms, confirming that fractions isolated differ mainly in carbohydrate content. Results of N-acetylglucosamine and neutral carbohydrate determinations are shown in table 4.
The results are consistent with the proposed nomenclature. The stepwise increment in the number of galactose and N-acetyl glucosamine residues is in concordance with the degree of branching of expected complex type glycans attached to particular subfractions. Integer number were not achieved which can not solely be ascribed to impurities in isolated fractions in view of observed ratios between carbohydrates. This claim is further substantiated by the additional carbohydrate determination on 4 and 5-sialotransferrin that had been purified by concanavalin A affinity chromatography before isoelectric focusing. The shift in N-acetylglucosamine and galactose in the direction of integer numbers is an indication that a small proportion of protein carrying 2 bi-antennary glycans but nevertheless 5 sialic acid residues exists.

Table 4. Carbohydrate composition of sialo-variants of human transferrin

After trifluoroacetic acid hydrolysis of isolated transferrrin subfractions neutral carbohydrates were determined in a single run on an adapted Multichrom M Analyzer with a 60 cm long column filled with Aminex A-6 resin. Data represent means of three determinations. Results are expressed as moles aminoacid per mole protein. N-acetyl glucosamine has been determined simultaneously with aminoacids. Sialic acids were determined according to the spectrophotometric method of Horgan[227].

Subfraction	Carbohydrate	Measured	Expected*
2 Sialotransferrin	N-Acetylglucosamine	7.6	8
	Mannose	5.1	6
	Galactose	3.7	4
	Sialic acid	2.1	2
3 Sialotransferrin	N-Acetylglucosamine	8.3	8
	Mannose	5.2	6
	Galactose	3.9	4
	Sialic acid	3.1	3
4 Sialotransferrin	N-Acetylglucosamine	8.2	8
	Mannose	5.6	6
	Galactose	4.1	4
	Sialic acid	3.9	4
5 Sialotransferrin	N-Acetylglucosamine	8.7	9
	Mannose	5.7	6
	Galactose	4.8	5
	Sialic acid	4.8	5
6 Sialotransferrin	N-Acetylglucosamine	9.4	10
	Mannose	6.0	6
	Galactose	4.7	6
	Sialic acid	5.8	6

* The expected value is based on the numbers to be found in transferrins carrying completed glycans. Two biantennary glycans contain 4 sialic acids, 4 galactose, 6 mannose and 8 N-acetyl glucosamine residues. The combination of a di- and a tri-antennary glycan will together contain 5 sialic acid, 5 galactose, 6 mannose and 9 N-acetyl- glucosamine residues.

In all fractions a small amount of glucose and fucose (0.2 mol/mol) was found. The glucose is most probably due to nonenzymatic ε-amino glycosylation of lysyl residues in the protein and this is not related to the N-linked glycosylation. Fucose can be part of N-linked glycans, particularly linkage to the N-acetylglucosamine in the common core structure has been described, but therefore it has no relation to variation in branching. As an independent variable it is of uncertain significance in transferrin. It was found in amounts equivalent to fucosylation of one in 10 glycans. Also nonenzymatic linkage to the protein moiety analogous to glucose has not been ruled out.

Table 5. Carbohydrate analysis of transferrin subfractions selected for glycan branching type by concanavalin A affinity.

Subfraction	Carbohydrate	Measured	Expected*
Bi-bi-antennary			
4 Sialotransferrin	N-Acetylglucosamine	8.3	8
	Mannose	6.0	6
	Galactose	4.1	4
Bi-tri-antennary			
5 Sialotransferrin	N-Acetylglucosamine	9.3	9
	Mannose	6.0	6
	Galactose	5.0	5

In conclusion, the assumption that transferrin subspecies that can be separated by isoelectric focusing represent proteins that differ only in the structure of N-linked glycans is supported by these data and permit interpretation of the pattern in the fashion described above (page 15 - 21).

Chapter 6.

Transferrin microheterogeneity in vivo.

Microheterogeneity patterns in vivo.

Identification of factors influencing microheterogeneity patterns.

Regulatory aspects of N-glycosylation and protein synthesis.

Hypothesis on the role and rationale of glycosylation changes.

Different degrees of sialylation and galactosylation of human transferrin in biological fluids such as cerebrospinal fluid, amniotic fluid and synovial fluid have been described[90,228]. The occurrence of a particularly high proportion of asialotransferrin in cerebrospinal fluid has been put to use in a clinical setting. Early diagnosis of cerebrospinal fluid leakage, most commonly presenting as otorrhea or rinorrhea, can be of vital importance since recurrent meningitis is one of the serious complications. Cerebrospinal otorrhea and rhinorrhea are notoriously difficult to diagnose, often requiring labour-intensive, costly radiographic procedures that are not without risk to the patient. By analysing the microheterogeneity pattern of suspected fluid and demonstration of the existence of a pronounced asialotransferrin band, the initial diagnosis cerebrospinal fluid leakage can be made[229].

Variation in the microheterogeneity pattern of transferrin can also be found or induced by several pathological and physiological conditions. A highly aberrant pattern has been described in children suffering from a rare syndrome dominated by neurological abnormalities[230,231]. The di- and asialofractions are present in high concentrations, a phenomenon which also occurs in the cerebrospinal fluid of these children. Not only transferrin is abberantly glycosylated in this condition. Other glycoproteins such as α_1 antitrypsin and ceruloplasmin have been found to be glycosylated differrently[232,233]. A hitherto not yet exactly defined metabolic error in the early steps of protein glycosylation leading to the synthesis of glycoproteins with reduced numbers of otherwise normally structured glycans is the basis of this disease, now known ass the carbohydrate deficient glycoprotein syndrome.

More subtle changes are induced in rheumatoid arthritis and idiopathic hemochromatosis. In sera from these groups an increased proportion of the highly sialylated transferrins and a

decreased proportion of tetra-and desialylated transferrins are found compared with healthy controls.

Microheterogeneity patterns in vivo.

In the following studies we have for the first time fully described microheterogeneity patterns observed in a number of groups of individuals, and quantitated these not only in relative amounts but also in actual concentrations of subfractions. The investigated groups are presented in table 6.

Table 6. Investigated groups.

Designation	Group Description	n
C	Control males	8
N	Control females	13
I	pregnant women, week 8-16	12
II	pregnant women, week 17-26	10
III	pregnant women, week 27-40	18
AT	pregnant women, a terme week 36-40	9
PP	3 days post partum	9
PPM	5 weeks postpartum	7
O	women on oral contraceptives	7
Fe	Iron deficiency anemia	9
RA-NA	Rheumatoid arthritis, not anemic	6
RA-Fe	Rheumatoid arthritis, iron deficiency	6
RA-ACD	Rheumatoid arthritis, anemia of the chronic diseases	6

The rationale for choosing this wide range of groups should be clear from the discussion as it was hoped that through the respective denominations of (patho-) physiologic circumstances both information on regulatory aspects of transferrin protein and glycan synthesis and information on the role of microheterogeneity in rion metabolism would be obtained.
In tables 7,8 and 9 results are given (standard deviations in accompanying tables in appendix A). Groups of patients have been designated as explained in table 6.
Between males and control females no significant differences existed in diferric transferrin spectrum, total and fractional transferrin concentrations.
Of a number of control male individuals repeated blood samples have been taken. These data show that there was very little variation in the spectrum of sialotransferrin variants in the course of 3 months (table 12).

Table 7 Variation of pattern in control male individuals. Microheterogeneity patterns were determined in four individuals 3 times in three months. The number of intra-individual comparisons possible thus amounts to 12 and the maximum inter-individual comparisons sums up to 54 combinations.

	transferrin sialo - variants (%)						
	mono	di	tri	tetra	penta	hexa	hepta
Average distribution (n = 4)	0.0	2.6	7.4	66.1	18.9	4.9	0.1
Maximum inter-individual variation (n = 4; 54 combinations)	0.0	1.1	2.1	2.6	1.5	1.9	0.1
Maximun intra-individual variation (n = 4; 12 combinations)	0.0	0.2	0.5	0.8	0.6	0.3	0.1

Besides confirming the recognized effect of pregnancy on total transferrin synthesis, our results showed a consistent, progressive redirection of synthesis towards highly sialylated transferrins in the course of pregnancy. This effect became statistically significant in the second trimester, and the trend appeared to be continued up to the last week of pregnancy. Compared to any of the other investigated groups pregnancy had the most pronounced effect on the microheterogeneity pattern. After delivery, changes were rapidly reversed. This was demonstrable as early as four days after delivery and 5 weeks later both concentrations and subfraction-pattern had virtually normalized (tables 8 and 9, figure 15). The trisialo-transferrin decreased significantly in its percentual representation, and the disialotransferrin was maintained at a constant level. Women using oral contraceptives displayed a similar trend: concentration of total transferrin was increased and compared with the proportional distribution in controls, more of the increase was accounted for by the highly sialylated variants. Compared to pregnancy it differred

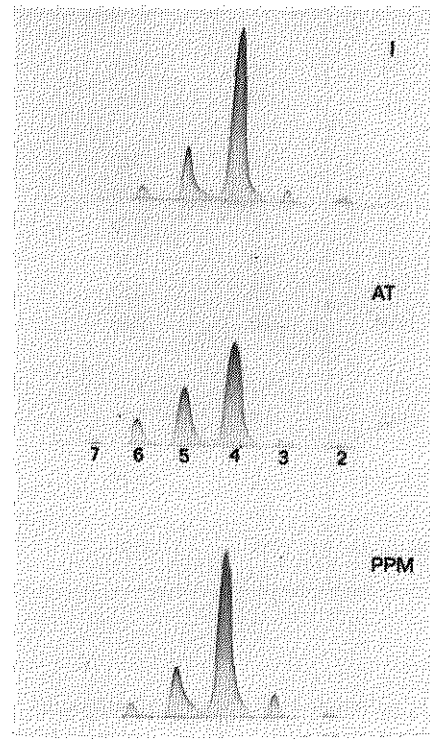


Figure15. Transferrin micro-heterogeneity in first trimester, at term and one month after delivery respectively.

in that at the same level of increment of total transferrin concentration, the change in the proportional representation was less marked than in pregnancy.
Iron deficiency was accompanied by an increased total transferrin concentration, the group average being risen by 48.2%. Relative proportions were shifted considerably less towards the highly sialylated fractions, reaching statistical significance only for the penta-sialotransferrin. Compared to pregnancy total transferrin concentration in iron deficiency was higher, and most of the actual increment in concentration was due to increased production of tetrasialotransferrin.

Table 8. Proportional distribution of transferrin subfractions in groups as defined in table 6. Distribution expressed as percentages of total amount. Statistical comparison of results with control; $p < 0.01$ indicated by *. Statistical comparison of results within RA groups to RA-ACD; $p < 0.01$ indicated by #.

Group	mono	di	tri	tetra	penta	hexa	hepta
M	0.0	2.5	7.3	66.3	18.9	4.8	0.1
C	0.0	2.3	7.7	67.9	17.7	4.4	0.1
I	0.0	2.6	8.5*	64.3*	19.5*	4.9	0.1
II	0.0	3.0*	6.7*	58.0*	24.6*	7.1*	0.6*
III	0.0	2.7*	5.8*	56.9*	24.8*	8.6*	1.1*
AT	0.0	3.0*	5.0*	57.2*	24.8*	8.9*	1.1*
PP	0.0	2.0	8.3	61.6*	23.0*	7.0*	0.3
PPM	0.0	2.6	6.7	64.5	20.4*	4.7	0.1
Fe	0.1	2.5	6.4	65.5	20.5*	4.7	0.4
OAC	0.0	2.7	6.7	63.1*	21.1*	6.3*	0.2
RA-NA	0.0	2.7#	6.9#	63.1*	20.9*,#	6.0*,#	0.4#
RA-Fe	0.0	2.4	6.3*	59.9*	23.4*	7.1*	0.7*
RA-ACD	0.0	2.0	4.0*	60.5*	24.4*	7.9*	1.1*

The patients with active rheumatoid arthritis generally had a lowered total transferrin concentration. The decrease was less in the subgroup with concomitant iron deficiency, but still significantly low compared to healthy controls. The microheterogeneity pattern was again changed in favour of the highly sialylated transferrins. This change was least pronounced in the nonanemic RA patients and most pronounced in the ACD-group, with the iron deficiency group occupying the intermediate position. The synthetic balance was shifted to the extent that in both

the iron deficiency and ACD-subgroups the concentrations of penta-, hexa- and heptasialotransferrins were not significantly depressed compared to controls, while the total transferrin concentration in these groups was diminished by 15.1 % and 38.8% respectively. The tetrasialotransferrin was significantly depressed in both proportional representation and in concentration in all RA subgroups, and a similar effect is observed for the di-and trisialo-transferrin.

Table 9. Concentrations of transferrin subfractions in groups as defined in table 6. Results in µmol/l. Statistical comparison of results with control; $p < 0.01$ indicated by *. Statistical comparison of results within RA groups to RA-ACD; $p < 0.01$ indicated by #.

Group	di	tri	tetra	penta	hexa	hepta	Total
M	0.9	2.7	24.6	7.1	1.8	0.0	37.1
C	0.9	3.0	26.4	6.8	1.7	0.0	38.9
I	1.0	3.1	23.8	7.2	1.8	0.0	36.9
II	1.4	3.2	27.6	11.7*	3.5*	0.3*	47.7*
III	1.4	3.0	29.2*	12.8*	4.5*	0.6*	51.7*
AT	1.8*	2.9	33.6*	14.5*	5.2*	0.7*	58.6*
PP	1.4	3.0	32.9*	12.4*	3.8*	0.2	53.7*
PPM	0.8	3.3	25.8	8.1	1.9	0.0	40.0
Fe	1.4	3.7*	37.8*	11.9*	2.7*	0.2	57.8*
OAC	1.4	3.4	31.6*	10.7*	3.2*	0.1	50.3*
RA-NA	0.8	2.0*,#	18.1*,#	6.0	1.7	0.1	28.8*
RA-Fe	0.8	2.0*	18.9*,#	7.2	2.2	0.1	31.3*,#
RA-ACD	0.5	1.0*	15.0*	6.1	2.0	0.3	24.8*

A global shift of synthesis towards highly sialylated and more branched chains in all conditions understates the information available from these results. Reviewing results in the context of contemporary understanding of both the underlying pathophysiological conditions and the mechanism from which microheterogeneity evolves disclosed additional conclusions.

Identification of factors influencing microheterogeneity patterns.

Results from pregnant women (tables 8 & 9, figures 15 & 16) clearly show adaptation of transferrin glycan synthesis in the course of pregnancy. The tetra-sialotransferrin concentration increases despite its proportional decrease, the major part of the increase in the total transferrin

concentration during pregnancy, however, is due to the increase in the concentration of highly sialylated transferrins. No significant change in the concentration of the desialylated transferrins is registered, which is consistent with an adequately functioning asialoglycoprotein scavenger system.

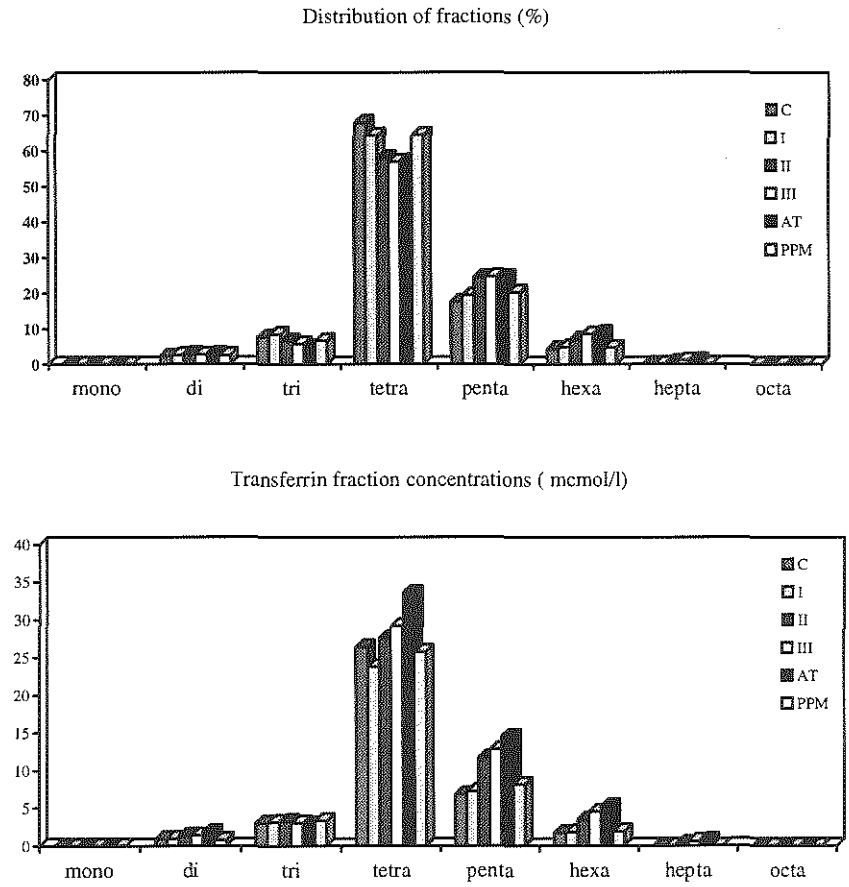


Figure 16. Transferrin microheterogeneity in the course of pregnancy. Numerals on X-axis indicate sialotransferrin fraction.

Endocrinological influences on the microheterogeneity pattern, were further probed comparing women on oral contraceptives and women suffering from an isolated iron-deficiency anemia with controls and pregnant women (fig 17). In women on oral contraceptives the pattern changes in the same direction as in pregnancy, as such confirming the notion that oral contraceptives can influence the glycosylation system with reference to transferrin[234]. Compared to pregnancy, at the same level of increment of total transferrin concentration, the change in proportional representation was less marked in women using oral contraceptives. This difference in pattern may be related to the difference in the estrogen to progesterone ratio as the

latter is higher in pregnant women than in the OAC group. Progesterone has been reported to influence glycosylation, data on estrogen are limited to stimulation of total transferrin synthesis. Therefore the above described difference could be explained on the basis of these assumptions. Alternative explanations lie in the preponderance of other hormones in pregnancy. Prolactin can probably be excluded in view of the rapid normalization of the microheterogeneity pattern after delivery in lactating women, but as such endocrinological influences are certainly significant.

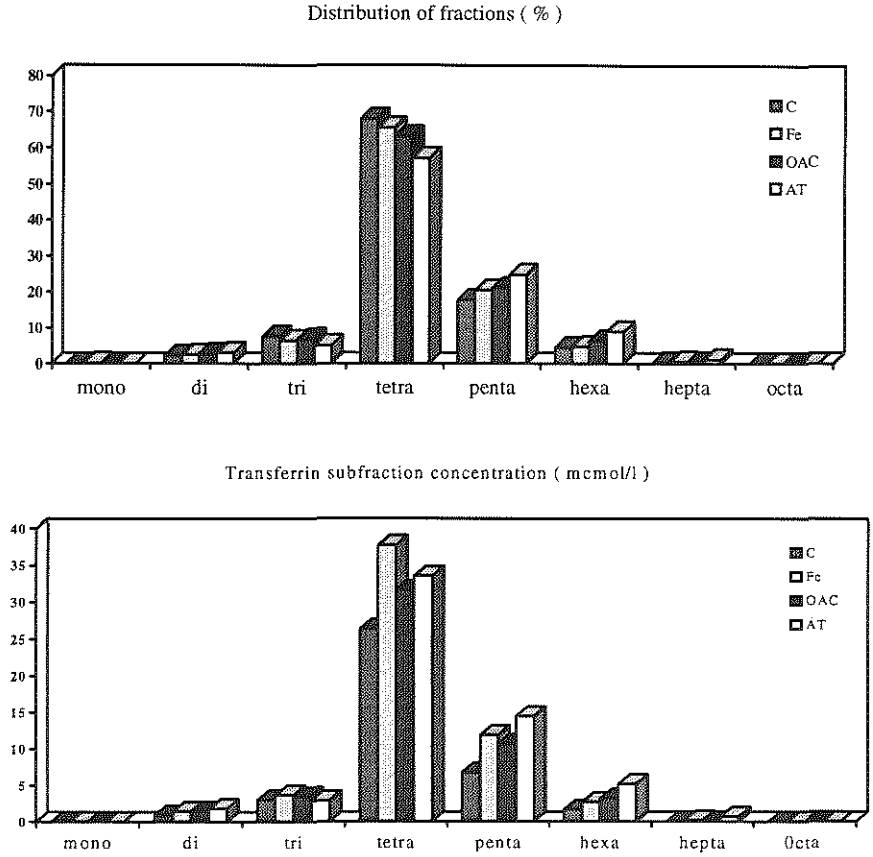


Figure 17. Transferrin microheterogeneity in control females, iron deficiency anemia, women using oral contraceptives and women at term pregnancy. Groups indicated as in table 6. Top: proportional distribution of sialotransferrin fractions on the left and corresponding concentrations on the right . Lower half: increments in relative amounts and actual concentrations respectively.

As reflected in clinical chemical parameters, the rheumatoid arthritis groups comprise a spectrum of disease activity[235,236]; in order of increasing disease activity the nonanaemic, the

iron deficient and the ACD group. Changes in microheterogeneity are again similar in direction, but this time protein synthesis is significantly reduced as a concomitant feature of the acute phase reaction. As the alteration in glycosylation was more pronounced in the anemic groups with the ACD group displaying the greatest change in pattern, change in glycosylation of transferrin in RA appears to be correlated with disease activity (figure 18), confirming conclusions drawn from studies on concanavalin A binding patterns of α_1 acid glycoprotein[237]. The decreased concentration of the disialo- and trisialotransferrin in RA is significant, and this also appears to correlate with disease activity. The latter can be interpreted as resulting from the activation of the asialoglycoprotein scavenging system in the acute phase reaction, thus working at higher efficiency. The higher avidity of this system as such for desialylated tri- and tetra-antennary glycans[238] may be an additional factor.

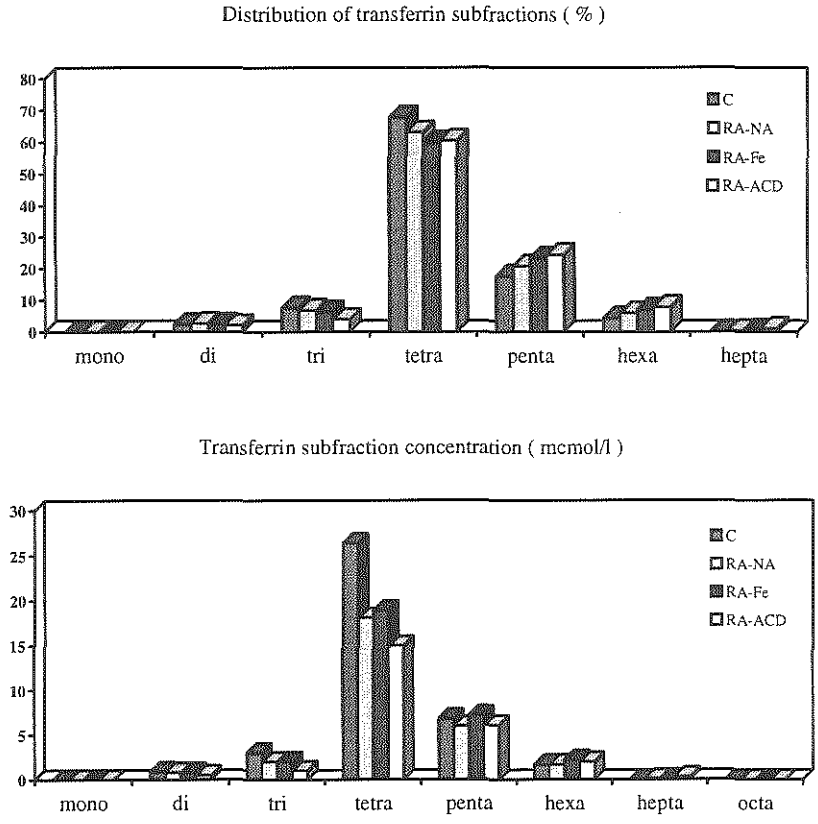


Figure 18. Transferrin microheterogeneity in control females and females with Rheumatoid Arthritis (RA) divided in three groups: non-anaemic RA, RA accompanied by iron defficiency and RA complicated by anemia of the chronic diseases. Groups indicated as in table 6. Top: proportional distribution of sialotransferrin fractions on the left and corresponding concentrations on the right . Lower half: increments in relative amounts and actual concentrations respectively.

Regulatory aspects of N-glycosylation and protein synthesis.

Evolutionary glycan synthesis is one step up the ladder compared to protein synthesis, particularly in its coding mechanism. Through both the possibility of branching and a combination of anomerous configurations and types of bonding between identical sets of components, a vast combination of structural determinants is created, as in other macromolecular synthetic machineries, but glycans are distinct from polypeptides and nucleic acids in the comparatively small size at which analogous astronomic diversity is attained. It is also distinct from nucleic acid and polypeptide synthesis in that it is not coded for by an accurate template mechanism. In essence glycan structure is coded for by intrinsic information laid down in the combination of primary, secondary and tertiary characteristics of the polypeptide core structure, in conjunction with characteristics of enzymes involved in the synthetic machinery as well as the interaction with the matrix in which it is processed. Consequently outcome is not explicitly defined, at the cost of loss of uniformity, hence the phenomenon of microheterogeneity.
From this understanding it evolved that amongst others the results of the isoelectric focusing of serum transferrin can be appreciated as a means of assessment of the precision of control over the glycosylation system. Our results are based on a technique that provides a very accurate and reproducible measure of transferrin microheterogeneity. In view of the complexity of the synthetic system and its control, it is clear how little variation there in fact is in the oligosaccharide-structures attached to this particular protein. Although some heterogeneity is still hidden from isoelectric focusing, only 9 different fractions can be distinguished in iron saturated human serum. As shown in table 2, between and within single individuals the variation in this microheterogeneity is minimal. Also in all groups with established disease / physiological states the pattern of distribution was similarly remarkable in its uniformity. Variation in microheterogeneity can therefore not just be the result of random variation. The consistency of isoelectric focusing patterns characterizes the system in a broad sense, demonstrating that the process must be governed by a very potent control system, although the mechanism, apart from identification of constituting elements, is still beyond our perception.
A second conclusion within the same context refers to the coupling between protein and glycan synthesis. Results on sera from pregnant women already indicate that protein core synthesis and change of glycosylation towards higher branched glycans are not linked, since the increment in the forms with tri- and tetra-antennary chains correlated better with duration of gestation than with concentration of total transferrin (figure 19). Women on oral contraceptives attain a lower level of change in microheterogeneity when compared to that stage in pregnancy where total transferrin concentrations are similar, which indicates that changes in microheterogeneity and transferrin protein synthesis are not linked.
The most persuasive argument is provided in the results from the RA-group. In acute and chronic inflammatory conditions increased synthesis of acute phase proteins is invariably accompanied by changes in the microheterogeneity spectrum, at the least not incompatible with a linkage. Transferrin being a negative acute phase protein[239], it provides the model in which the effect of protein synthetic rate on N-glycosylation is dissociated from the effect of the acute phase response on glycosylation. The microheterogeneity pattern can be changed markedly even when protein synthesis is significantly depressed. Therefore a fixed linkage between transferrin protein synthetic rate and glycosylation pattern can be denied.

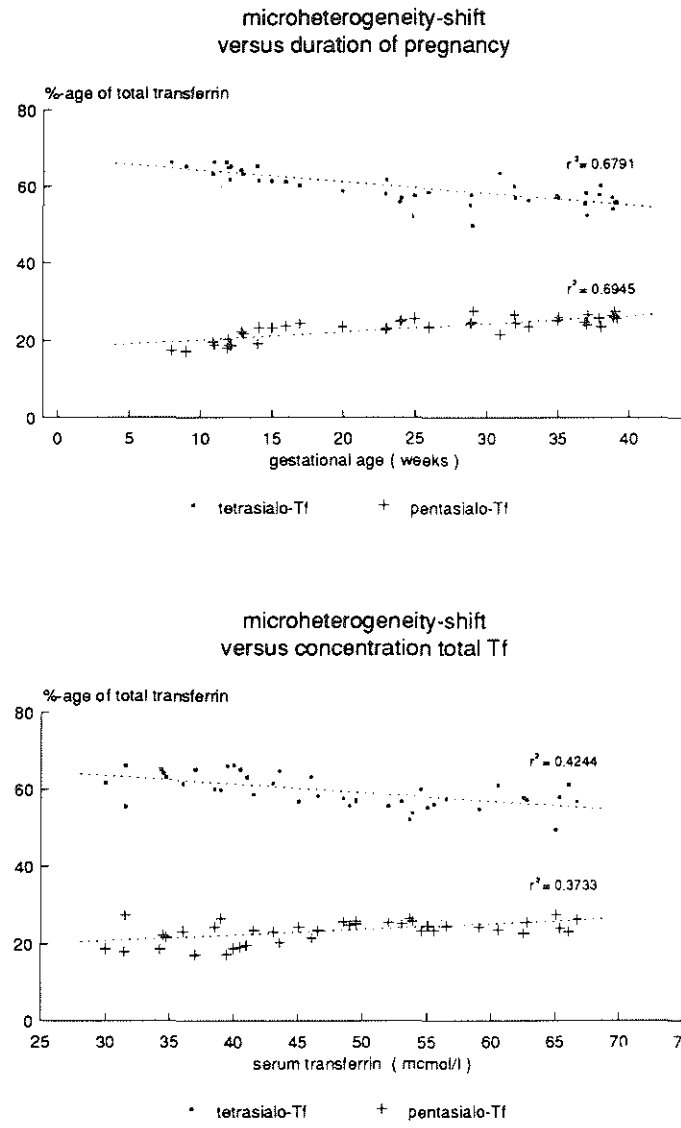


Figure 19. Correlation diagrams displaying redirection of transferrin glycan synthesis related to duration of pregnancy (top) and to total transferrin concentration (bottom). The difference in the correlation to duration is significantly higher than the correlation to total transferrin concentration; $p < 0.001$.

Hypothesis on the role and rationale of glycosylation changes in relation to physiological and pathophysiological requirements for iron.

Having concluded that observed changes in tranferrin microheterogeneity are not just related to random faults or a byproduct of increased synthetic rate, but the result of strictly controlled redirection of glycan synthesis, the next question that arises is whether these changes in transferrin have any functional significance, or is transferrin simply an innocent bystander as it happens to pass the synthetic machinery that has been adjusted for other reasons. To address this question results are reviewed in their pathophysiological contexts.

Pregnancy

The adaptation of the microheterogeneity pattern in the course of pregnancy in timing closely coincides with the major changes in iron metabolism as they occur during pregnancy. The increased demand of the fetoplacental unit and maternal bone marrow for iron is partly counterbalanced by cessation of menstruation. The remainder is partly met by increased iron absorption from the gut. The cumulated demand for iron may increase to above 8 mg/day, which exceeds the uptake rate from the gut. Therefore mobilization of iron from storage sites is an important mechanism in pregnancy, particularly in the later stages. The correlation between the increment in the production of highly sialylated transferrins and combined daily maternal and fetoplacental iron needs, combined with the absence of correlation with the production of tetrasialotransferrin, is striking (figure 20). As both major iron requiring processes, i.e. maternal erythroblast iron uptake and iron uptake on the maternal side of the placenta have been shown to be dependent on the interaction between transferrin and the transferrin receptor, the obvious conclusion is to relate these changes in microheterogeneity to increased iron fluxes. This concept of redirection of iron fluxes through modulation of functional characteristics of the molecule without affecting the protein moiety and independent from its rate of production is one that has a certain attraction, but admitted, coincidental concurrence can certainly not be excluded and the concept must await experimental verification. However, some additional weight is added to the argument by observations in other diseases (see paragraphs on iron deficiency anemia and RA).

Iron deficiency anemia

Iron deficiency, if simply defined, is a state of global physical demand for iron in the organism. Having postulated a functional role for the change in glycosylation in iron metabolism via modification of functional characteristics of transferrin a dilemma is thus created: what rationale is there to account for the fact that changes in obviously the pre-eminently iron demanding condition are less prominent than in pregnancy ?
The answer may lie in both the nature and the site of generation of the signal to adapt to the stress the organism is subjected to in each particular condition. In iron-deficiency the matter is above all to absorb more iron from the gut, and not such much to re-distribute as to distribute any of the available iron as all tissues are devoid of iron. Transferrin plays no direct active role in absorption of iron from the intestines, denying a role for changes in transferrin-substructure in it as well. Secondly, the single most important site of production of transferrin that ends up in the circulation is the liver, which controls transferrin protein synthesis. During pregnancy in essence the fetus, through regulation of the hormonal milieu in concert with the placenta,

controls the whole range of maternal metabolic, hematologic and even cardiovascular adaptations to pregnancy[240]. Regardless of the presence of anemia increased erythropoiesis is forced upon the mother, dictated by the fetoplacental unit. Even more so than in iron deficiency iron must be lured away from storage sites or preferentially be taken up by organs other than those involved in storage, explaining the logic for a physiological mechanism different to that in iron deficiency anemia.

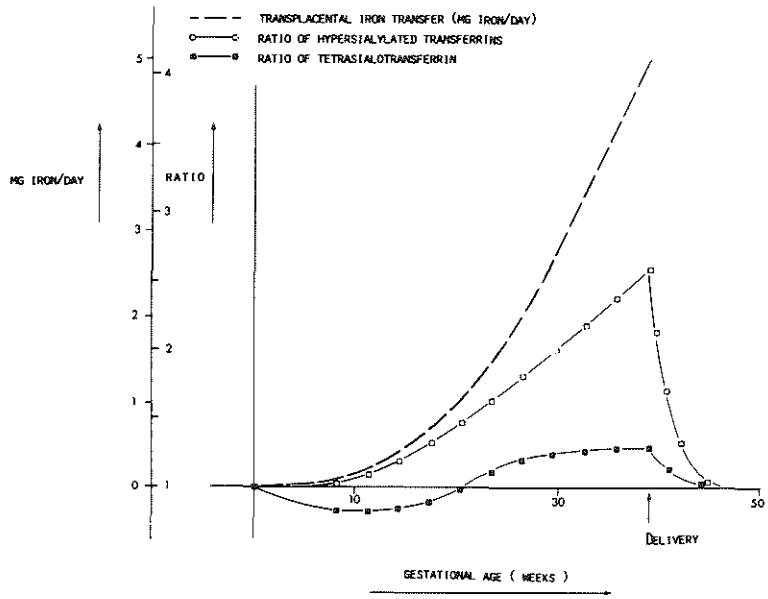


Figure 20. Transferrin microheterogeneity in relation to transplacental iron transport during pregnancy . (------) Transplacental iron transport in mg/day (estimated figures based on data on transplacental iron transport[241]). (□——□) Pentasialotransferrin production ratio. (■——■) Tetrasialotransferrin production ratio. Production ratio's are estimates from the ratio of actual subfraction concentration over initial subfraction concentration multiplied by a correction factor for increased vascular mass, assuming unaltered serum half lives of transferrins in the course of pregnancy.

Rheumatoid arthritis

Referring to the relative conservation of the production of highly sialylated transferrins, the above postulated facilitation of iron fluxes to the bone marrow through transferrin glycan modulation can also be incorporated in the interpretation with respect to rheumatoid arthritis. In view of the high correlation of disease activity and shift in microheterogeneity pattern, the conservation of highly branched glycans may form part of a compensatory mechanism in

anemias accompanying rheumatoid arthritis, attempting to facilitate a decreased erythroblast iron availability which has been postulated to be a major contributing factor in this condition. This decreased iron availability may rely on inhibitory effects of cytokines such as the interleukins and TNF, factors that have been shown to correlate highly with disease activity[240,242]. The same mechanism would of course also operate in the disease stages not accompanied by ACD, but then be compensated for. In ACD this compensation appears to finally fall short. Of course this notion is based on correlations only and one could just as reasonably turn the argument around. For instance it can be postulated that anemia of the chronic diseases arises when the tetra-sialotransferrin concentration falls below a critical level.

Chapter 7.

Microheterogeneity *in vitro.*

Binding of purified microheterogeneous forms of transferrin to the transferrin receptor on placental microvillous transferrin receptors

Uptake of iron from microheterogeneous forms of transferrin by a liver cell line

Uptake of iron from microheterogeneous forms of transferrin by human bone marrow

Iron binding by microheterogeneous forms of transferrin

Transmembranous NADH diferric transferrin reductase activity in relation to microheterogeneity

Recombinant nonglycosylated transferrin

From the inventory of the glycosylation patterns in the groups described above, it followed that changes in these pattern might be instrumental in modulation of iron metabolism. To further assess this possibility in vitro experiments were devised in keeping with the several functions known for transferrin:

1. Transport-function; the three quantitatively most important target organs have been tested
    a. Binding to placental membrane transferrin receptors.
    b. Transferrin and iron uptake by liver cells.
    c. Iron uptake by bone marrow.

2. Affinity for iron.

3. Transferrin receptor associated NADH-reductase activation in liver cells.

The bacteriostatic effect of the microheterogeneous forms of transferrin has not been compared.

As this is related to the chelating properties of the protein this will most probably be changed analogous to changes in iron affinity. Changes in prevention of spontaneous oxygen radical formation are highly unlikely since glycans can in no way influence the conformation of iron binding structures within the two domains.
These topics are discussed in the above indicated sequence; an integration of findings and the interpretation within the context of iron metabolism is presented in chapter 8.

Binding of purified microheterogeneous forms of transferrin to the transferrin receptor on placental microvillous transferrin receptors

From human term placenta microvilli, vesicles with normal membrane orientation carrying transferrin receptors were isolated. Transferrin-receptors isolated can be expected to be saturated with maternal transferrin. A choatrope treatment therefore preceeded the usual isolation procedure before incubation with transferrin samples of interest.
Membranes were incubated with either iodine-labeled 4 sialotransferrin or with iodine-labeled 6-sialo transferrin. Both transferrins were fully iron saturated and in all experiments correction for nonspecific binding was performed in a simultaneous run in 200-fold excess of unlabeled diferric transferrin.
In figure 21 results are shown in the Scatchard representation.

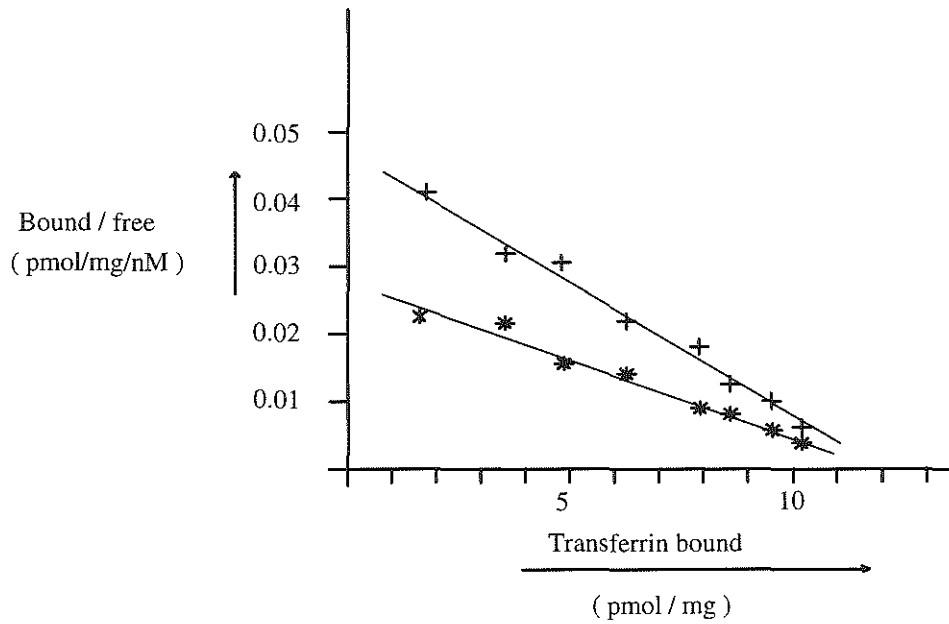


Figure 21. Scatchard plot of binding data. Comparison between 4-sialo (+) and 6 sialotransferrin (*).

Table 10. Binding of transferrin subfractions to human placental microvilli transferrin receptor.

transferrin fraction	K_a ± s.e.m. (M^{-1}) • 10^7	Tf R / mg pellet protein • 10^{12}
4 sialotransferrin	2.4 ± 0.3	3.8 ± 0.4
6 sialotransferrin	1.4 ± 0.8	3.6 ± 0.3

Association constants, number of binding sites and nonspecific binding were calculated from the data by nonlinear least squares analysis applying the Marquardt-Levenberg algorithm.

Uptake of iron from microheterogeneous forms of transferrin by a liver cell line

For determining the influence of microheterogeneity on iron uptake by liver cells in practice the choice is limited between isolating heterologous noncarcinomatous hepatocytes from perfused livers and homologous carcinomatous cell cultures. The initial choice for the human liver cancer derived cell line HepG2 is based on the preference for using a homologous model[243] plus the fact that the HepG2 cell line expresses both major receptor systems involved in transferrin uptake in liver cells, i.e. the transferrin receptor and the asialoglycoprotein receptor system.
HepG2-cells grown to confluent state in culture flasks were incubated with microheterogeneous forms of transferrin in the diferric form. Transferrin subfractions used were isolated by isoelectric focusing without prior separation on a concanavalin A column. Therefore heterogeneity within these fractions still present from partially desialylated forms has to be incalculated (e.g. 5-sialotransferrin having lost one sialic acid residue in the 4-sialo fraction etcetera).

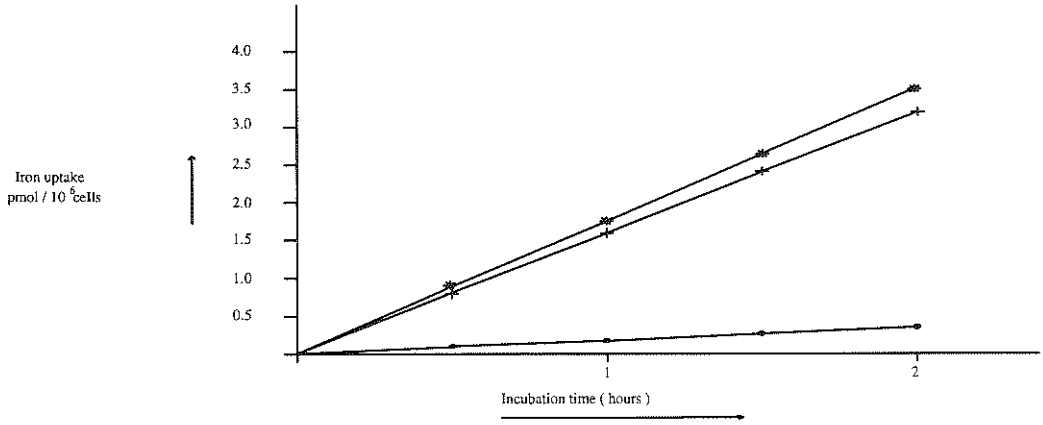


Figure 22. Iron uptake from 4-sialotransferrin by HepG2 cells. (*) total uptake, (+) TfR-uptake and (•) ASGPR-uptake.

In all experiments transferrins used were added to a concentration of 0,3 µmol/l, which is known to provide saturation of the transferrin receptor with respect to the receptor mediated endocytosis of transferrin.
In retrospect a higher concentration might have been a better choice since the transferrin receptor asssociated oxidoreductase reaches saturation at 4 µmol/l. The small amount of transferrin available at the time restricted possibilities in that respect however, so that conclusions referring to NADH-reductase activity have been tested independently at a later stage.
The total iron uptake from 4 Sialo-transferrin in the course of two hours incubation is shown in figure 22.
For all fractions examined, correction for nonspecific uptake by subtraction of results obtained in presence of 200-fold excess unlabeled transferrin; transferrin-receptor mediated uptake was determined by including asialofetuin in the incubation-medium which also predicts the asialoglycoprotein receptor-mediated uptake.
Identical time courses were found for the other transferrin fractions tested. The summary of the data obtained with all fractions is given in table 11. These are based on the determination of internalized iron after 2 hours of incubation at 37 °C, and all means of experiments in triplicate.

Table 11. Iron uptake from transferrin by HepG2 cells (pmol Fe / 10^6 cells / hr ; results are means of three experiments) ASGPR = asialo-glycoprotein receptor.

Transferrin fraction	total uptake	Tf R-mediated uptake	ASGPR-mediated uptake	percentage uptake of total via ASGPR
2 Sialo-transferrin	1.43	1.18	0.25	17.5 %
4 Sialo-transferrin	1.77	1.60	0.17	9.6 %
5 Sialo-transferrin	1.69	1.54	0.15	8.8 %
6 Sialo-transferrin	1.55	1.48	0.07	4.5 %

Uptake of iron from microheterogeneous forms of transferrin by human bone marrow

Human bone marrow was incubated with ^{59}Fe-labeled diferric 4 Sialo- or 6 Sialo-transferrin. The transferrin concentration in the incubation medium was 1 µmol/l. Uptake was linear over the observed period of time. The internalization of iron from 6 Sialo-transferrin was equally effective.

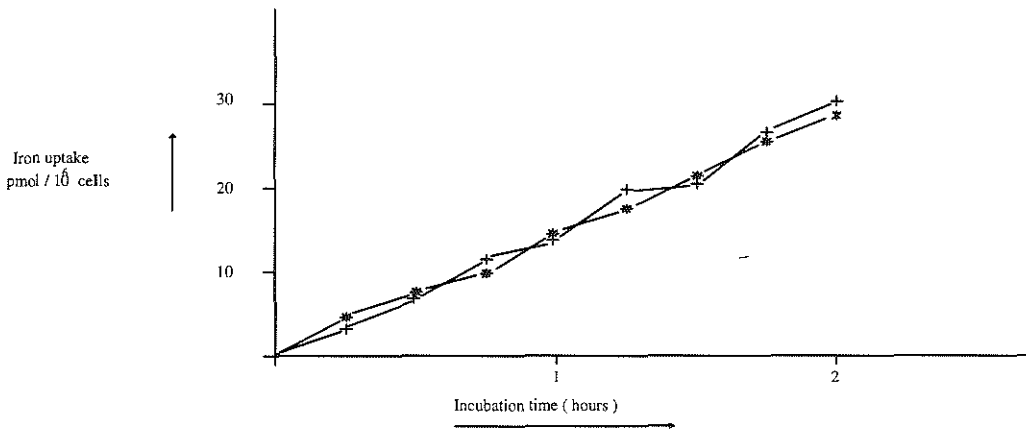


Figure 23. Iron uptake by human bone marrow cells from 4 sialotransferrin (+) and 6-sialotransferrin (*).

Iron binding by microheterogeneous forms of transferrin

Equilibration dialysis of 4 and 6 Sialotransferrin was performed according to Aasa et al[244]. Fractions were left to equilibrate for 5 days.
The 6 Sialotransferrin displayed an affinity for ferric iron equal to that of the 4 Sialotransferrin. This is in agreement with the prediction based on the molecular chemistry of transferrins[245].

Transmembranous NADH diferric transferrin reductase activity in relation to microheterogeneity

Hepatocytes prepared from Wistar rats by regional perfusion of the liver with collagenase IV via the portal vein, followed by excision of the liver and isolation of hepatocytes. Viability as tested by the trypan Blue exclusion test was 90 %. Hepatocytes were incubated with 5 µM diferric transferrin solutions and iron transfer to bathophenantroline was measured through the absorbance change at 535 nm and 4-sialo diferric transferrin was compared to 5-sialotransferrin.

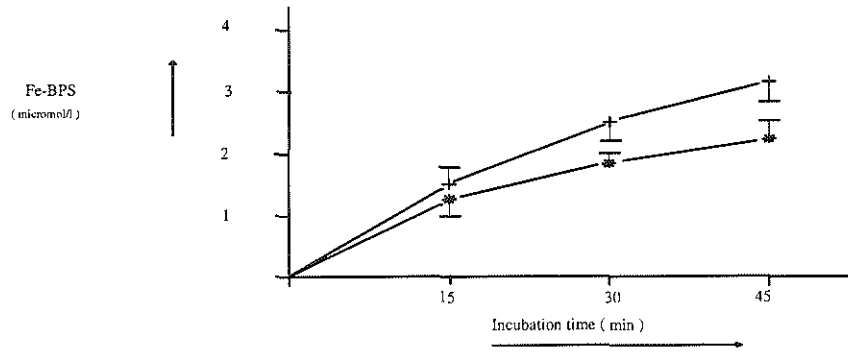


Figure 24. Formation of bathophenantroline disulphonate catalyzed by intact rat hepatocytes in the presence of 4-sialotransferrin (+) and 5-sialotransferrin (*).

A simultaneously performed uptake study identical to the experiment with HepG2 cells was performed on isolated rat hepatocytes, employing ^{59}Fe-labeled diferric 4-sialo bi-bi-antennary and 6-sialo tri-tri-antennary transferrin. After a 2 hour incubation hepatocytes had shown a prefererence for uptake from 4-sialotransferrin compared to the 6-sialotransferrin (table 12).

Table 12. ^{59}Iron uptake from transferrin by rat hepatocytes (pmol Fe / 10^6 cells / hr).

Transferrin fraction	total uptake	Tf R-mediated uptake	ASGPR-mediated uptake	% uptake of total via ASGPR
4 Sialo-transferrin	1.17	1.11	0.06	5.1 %
6 Sialo-transferrin	1.02	0.98	0.04	3.9 %

The uptake via the asialoglycoprotein receptor compared to the results obtained in HepG2 cells is lower. Probably the exhaustive purification of the transferrins used in this experiment accounts for this difference.

Recombinant nonglycosylated transferrin

As a further step towards the understanding of the function of glycans in transferrin chemistry and in transferrin metabolism, we decided to study the biochemical, structural and physico-chemical properties of human transferrin completely lacking the carbohydrate moiety. Attempts to enzymatically remove the glycans employing endoglucosidases either failed or rendered a denatured endproduct that could not be renatured, or the sugars were only partially removed. Similar results were obtained employing HF and various other chemical methods.
Bacteria lack the Golgi system and therefore N-linked glycans are not found in bacterial products. Also if we managed to get the transferrin expressed in a bacterial expression system at low rates, conceivable upscaling of production would be comparatively easy in view of the simple nutritial demands of bacteria, as opposed to continuous cell lines.
In collaboration with us Dr M.H. de Smit from our depatment has been able to isolate 3 E. coli strains endowed with plasmids containing cDNA's coding for either human serum transferrin, the N-terminal or the C-terminal half transferrin. Furthermore, the authentic amino acid sequence has been maintained[246]. Induction of plasmid expression by the thermoinducible P_L-promotor gave rise to low yields. This promotor was initially chosen because in the uninduced state it will be 99 % repressed. Strict control of expression was considered essential because the bacteriostatic potential of transferrin, the plasmid carrying the cDNA might turn out to be a Trojan Horse to the E. coli. Indeed a bacteriostatic effect is registered upon induction of expression but this is probably of limited importance since the bacterium appears to be able to acquired enough iron from the medium to saturate the protein and thus neutralise this negative effect.

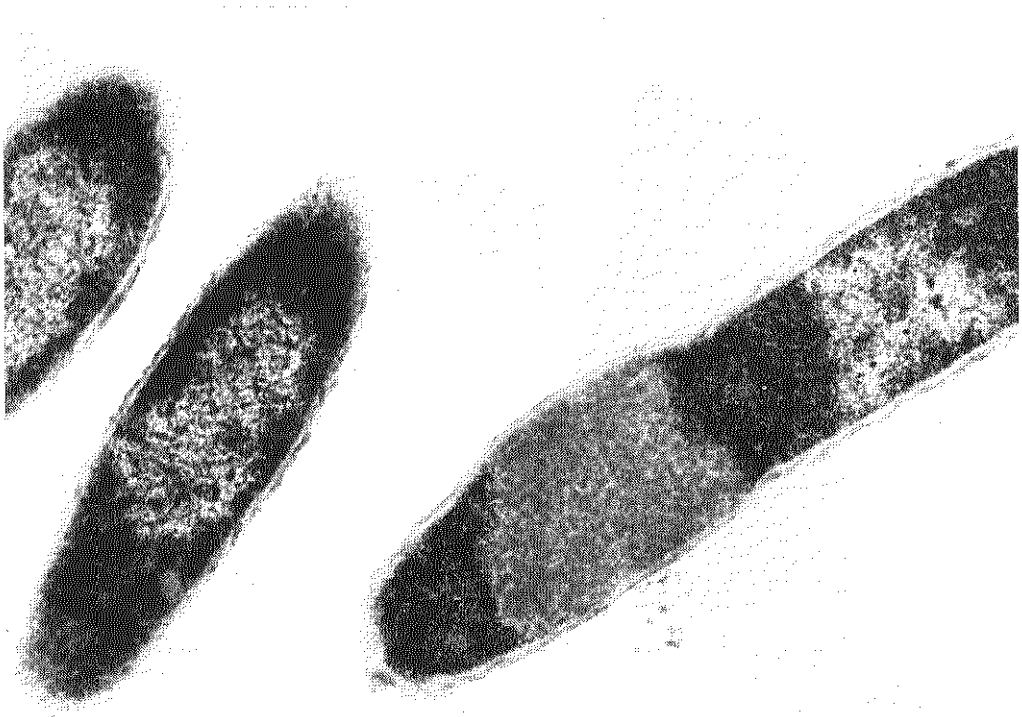


Figure 25. Formation of inclusion bodies containing human a-glycotransferrin in induced E.coli LC137.

The induction method proved to be more problematic. The temperature shift to 42 °C necessary for induction of the P_L-promotor, most likely through the heat shock response resulting in increased expression of chaperonins and proteases due to increased protein turnover transferrin yields remained low. After switching to the P_{tac} promotor which is chemically inducible at 28 ° C, thus avoiding the problems related to the heat shock response, and use of the protease deficient E. coli strain LC137, production was stepped up to 60 mg of nonglycosylated transferrin per litre of bacterial culture.

The interior of the bacterium more closely reflects the circumstances that prevailed in the reducing atmosphere that existed when life began some 3.5 billion years ago. As a result bacteria, in contrast to eucaryotic cells that arose in aerobic circumstances,, besides not glycosylating proteins, also do not form disulphide bridges. Large proteins such as transferrin that can form 19 disulphide bridges will therefore be devoid of this important stabilizing mechanism. As a result, but also due to the action of bacterial chaperonins, the protein is not found in the cell in its native conformation but in a denatured state in large amorphous particles called inclusion bodies (figure 25). After cell destruction by sonification more than 95 % of the recombinant protein is found in the insoluble fraction derived from these inclusion bodies. The protein has to be liberated from these bodies and to this aim it first needs to be denatured even further by means of dissolution in 8 M urea and reduction of disulphide bridges by dithiothreitol (appendix A). Under these reducing conditions the protein can be elctrophoretically separated from the bacterial proteins and we have been able to obtain the purified recombinant nonglycosylated human transferrin and its half molecules, as is shown clearly by the SDS-PAGE (figure 26) and the amino acid analysis (table 13).

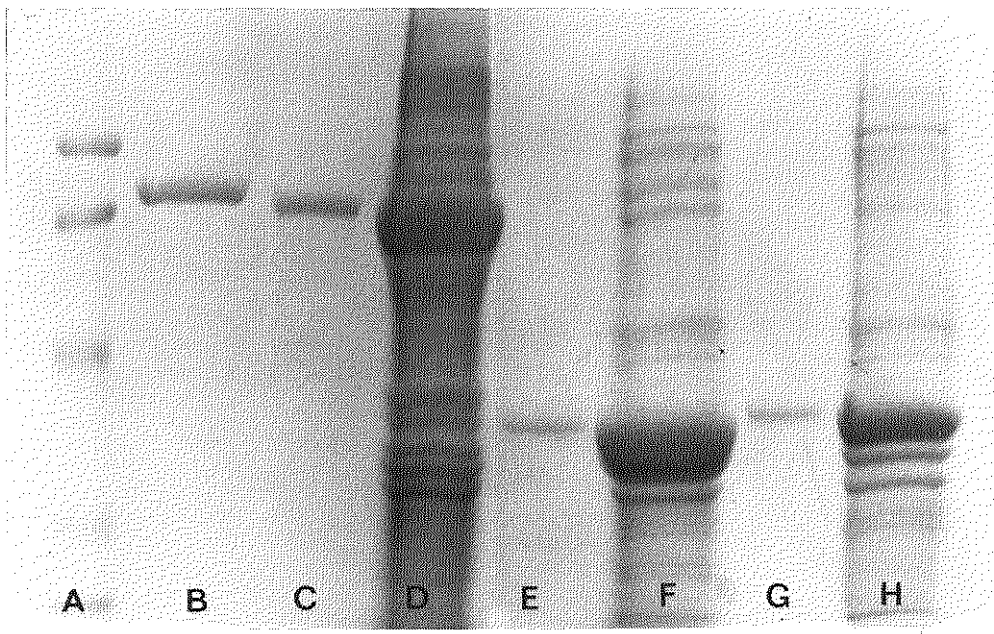


Figure 26. SDS PAGE of native transferrin and recombinant transferrins isolated from E.coli LC137. A: molecular weight markers. B: native human transferrin. C: recombinant human aglycotransferrin. D: bacterial lysate from which complete recombinant protein was isolated. E: recombinant N-terminal half transferrin. F: Lysate from which E was isolated. G: C-terminal recombinant half transferrin. H: correponding bacterial lysate.

Apart from acknowledging the supposition, based on the structure of the plasmid, that the authentic amino acid sequence has been maintained, the analysis also showed no detectable N-acetylglucosamine and a carbohydrate analysis performed independently confirmed the nonlycosylated state of the protein. Upon induction of the recombinant transferrin in E. coli at 28 °C (but not at 42 °C) the cell suspension develops a conspicuous brownish pink hue that resembles the salmon pink of diferric transferrin solutions. Although this is not conclusive as evidence that in its denatured state the recombinant transferrin is capable of binding iron, it does hint at the integrity of the iron binding site under these conditions. In the ensuing isolation procedure the protein is completely denatured and with it the capacity to specifically bind iron with high affinity is lost.

Table 13. Amino acid analysis of recombinant transferrins (mol aminoacid / mol protein). Sums of Asparagine and Aspartate and sums of Glutamine and Glutamate are given because of interconversion of these amino acids in the hydrolysis procedure. The colour reactant employed yields low absorbance products for Proline and Cysteine and these were thus not determined (n.d.). % deviation represents the cumulated difference between integer numbers of separate amino acids in the transferrin species compared to the reference values derived from figures based on the cDNA characterization by Yang et al[31].

	recombinant human transferrin (rTf)	reference values serum Tf	N terminal rTf	reference values serum Tf	C-terminal rTf	reference values serum Tf
Lysine	53.7	58	25.9	27	26.9	31
Histidine	18.0	19	9.2	9	8.7	10
Arginine	24.9	26	10.6	12	14.7	14
Aspartate+NH_2	74.0	79	35.3	24 + 12	42.2	21 + 22
Threonine	30.2	30	12.9	13	16.3	17
Serine	40.4	41	20.2	21	20.6	20
Glutamate+NH_2	57.8	59	28.5	18 +11	32.4	25 + 5
Proline	n.d.	32	n.d.	20	n.d.	12
Glycine	51.5	50	25.2	24	27.3	26
Alanine	58.4	57	31.7	32	25.3	25
Cysteine	n.d.	38	n.d.	16	n.d.	22
Valine	44.7	45	22.7	22	22.3	23
Methionine	9.4	9	5.8	5	5.3	4
Isoleucine	14.6	15	7.8	8	7.6	7
Leucine	55.2	59	30.7	29	29.5	30
Tyrosine	26.0	26	15.2	14	12.0	12
Phenylalanine	25.8	28	15.4	16	13.1	12
Tryptophan	7.5	8	2.9	3	4.2	5
Total		679		336		343
Total minus Cys and minus Pro	592.1	609	300.0	300	308.4	309
% deviation from predicted values	3.6 %		3.3 %		5.5 %	

Presently the renaturation procedure described in appendix A has yielded a water soluble product at a concentration of 3 µg / litre, capable of retaining iron with high affinity after equilibrium dialysis. In view of the very low protein concentration a reliable estimation of the affinity constant cannot be given yet. We did establish that this iron was retained after dialysis against PBS pH 7,4, and that mobilisation followed quickly after mild acidification of the transferrin solution. Also the renatured protein reacts with polyclonal rabbit anti human transferrin, at the least indicative of restoration of some of its epitopes to the native state.
The cells containing the N-terminal half transferrin cDNA also display the brownish pink discoloration after induction of protein synthesis, and protein isolated reacts with the polyclonal anti transferrin. The C-terminal half transferrin is also capable of reacting with the antibody, but that is presently the limit of our knowledge about these intriguing substances.

Chapter 8.

Synthesis of structure - function relationships

To what purpose are N-linked glycans present on transferrin. This has essentially been the issue we have been addressing.
Previous studies had failed to identify a specific role for these structures. Clearance of aged transferrin molecules by the liver was the only function identified for the glycans. This specific routing to the liver is dependent on eventual desialylation of glycans with subsequent clearance via the asialoglycoprotein receptor system which is present on hepatocytes only. The pluripotent glycoprotein scavenging role of this receptor is currently being criticised. Direct evidence for such a role is not available. Probably more specific, physiologic ligands exist[234]. With respect to transferrin metabolism, this receptor system has been overvalued in the past years. The asialoglycoprotein receptor system does not play a quantitatively important role in iron transport in healthy people. Firstly, the dissociation constant of the asialoglycoprotein receptor for bi-antennary chains is 10^{-6}M, indicating that under physiological conditions nonsaturating concentrations of desialylated transferrins will prevail. Secondly this system does not select for iron containing transferrin molecules, so at least two thirds of transferrin taken up in this manner will have been apotransferrin. Thirdly many of the other circulating glycoproteins and lipoproteins predominantly carry tri-antennary glycans, often more than three per molecule. These glycoproteins are present in high concentrations compared to the transferrins carrying tri- and tetra-antennary glycans. Although the affinity of the lectin for the tri-antennary chains is higher, with a dissociation constant of 10^{-9}M, the relatively high concentrations of physiologic ligands will effectively diminish the importance of this receptor system for normal iron uptake. Furthermore transferrin is predominantly utilised in the bone marrow. The number of endocytotic cycles a molecule can endure being limited, it is conceivable that most of the transferrin will be catabolised in the bone marrow. Finally, employing carefully selected transferrin species even in vitro the asialoglycoprotein route is of little importance (table 13), further substantiating the notion that the latter system is of little importance in iron metabolism. Thus, having stripped glycans of their alleged function in facilitating glycoprotein scavenging, one might conclude that for transferrin glycans, and by the same token changes in transferrin microheterogeneity, are of little physiological relevance.

An interesting fact is that one of the other major transport proteins, albumin, is not glycosylated at all. However, individuals synthesising N-glycosylated albumin have been detected by chance in healthy volunteer populations. Analysis of their albumin gene sequence has revealed that in these rare individuals a single site mutation has created an N-glycosylation site not present on normal albumin. Nevertheless, no difference in its transport function could be detected.

Albumin, as opposed to transferrin, is an inert nonspecific carrier. Selection for nonglycosylated protein in the case of albumin will be rewarded with a decrease in cellular energy expenditure as the building of glycans is a highly exergonic process. Despite this possible advantage, transferrin in all animal species examined carries at least one glycan per molecule, suggesting some evolutionary advantage must exist in relation to one of the physiological functions of transferrin.
As described in previous chapters, our approach has been to first study the microheterogeneity patterns *in vivo*. Our results indicate that changes in these cannot just be attributed to random faults in the complex coding system or to increases in synthetic rates. Instead changes in microheterogeneity are the result of a strictly controlled redirection of glycan synthesis. In view of the concurrence of changes in transferrin glycan structure and transplacental transport, and the relative conservation of the transferrin with highly branched glycans in rheumatoid arthritis, redistribution of body iron stores to bone marrow and placenta as a result of changes in the microheterogeneity pattern of transferrin was considered a possible mechanism.

On first inspection experimental results do not appear to favour this concept. Compared to the normally predominant form those transferrins becoming more abundant during pregnancy have lower affinity for the transferrin receptor on placental microvillous membrane, both in humans (table 10) and in the guinea pig[247]. Although *in vitro* the affinity for the transferrin receptor decreases by a significant 42 %, a shift towards the 6 sialotransferrin will not lead to decreased placental or bone marrow iron uptake. *In vivo* no difference will exist because the receptor affinity is in such an order of magnitude that it will at prevailing diferric transferrin concentrations ensure constant full occupancy of exposed transferrin receptors. Also, endocytosis and exocytosis rates of diverse transferrins have been shown to be identical[247]. With this established, van Dijk et al were able to show that in cells relying on receptor mediated endocytosis for iron uptake, once bound to its receptor, all microheterogeneous forms of transferrin will behave identical with respect to iron donation.
Finally, particularly with respect to pregnancy and other conditions accompanied by similar changes in glycosylation, the role of the transferrin receptor itself must also be taken into consideration. The observed affinity constants for the placental transferrin receptor are higher than values reported for reticulocytes. Possibly, this is due to changes in the end processing of the 3 N-linked glycans of the transferrin receptor itself as it has been shown that this influences the affinity of the placental receptor for transferrin[248]. Increased branching of glycans results in increased affinity of the receptor for its ligand, and this intrinsically compensates for the negative effect of the simultaneously changed glycosylation of transferrin. Whether the same applies to maternal bone marrow cells in the course of pregnancy remains to be investigated. The latter may be plausible or even likely, but this interpretation should certainly be verified, amongst others because organ specific differences in glycosylation exist that may be additional factors determining receptor ligand interaction. For instance, human placental glycoproteins contain only the Sia $\alpha 2 \rightarrow 3$ Gal linkage whereas in serum glycoproteins both the Sia $\alpha 6 \rightarrow 3$ Gal linkage or the combination of Sia $\alpha 6 \rightarrow 3$ Gal and Sia $\alpha 2 \rightarrow 3$ Gal linkages are found[98].
In summary, facilitation of iron fluxes to the bone marrow or placenta due to preferential or faster uptake from transferrin at these sites is not present. Upregulation of transferrin receptor density in target organs as a result of hormonal influences is an additional possibility, and this may indeed be present, but this alone would not explain redistribution of iron stores as this must include mobilisation of iron from intracellular ferritin. A similar argument holds for iron mobilisation in conditions leading to iron deficiency, and these are often not even accompanied

by changes in transferrin microheterogeneity. The body of cells constituting the iron stores would thus seem to play a pivotal role in this, and as most of the iron stores are present in the hepatocytes, iron uptake from various transferrin subfractions was studied in detail.

The liver cell iron uptake from transferrin is dependent on both specific uptake via the transferrin receptor and specific uptake via the asialoglycoprotein receptor. Uptake via the asialoglycoprotein receptor does not play a quantitatively important role in physiological iron homeostasis, transferrin receptor mediated iron uptake is therefore decisive in the interaction.
We have established that transferrin microheterogeneity does influence hepatocellular iron uptake form transferrin. In a liver carcinoma cell line we found a 7.5 % difference between 4 sialo and 6 sialotransferrin mediated iron uptake via the transferrin receptor, and an intermediate value was found for the 5 sialotransferrin. In isolated rat hepatocytes the comparable figure was 11 % as assessed by the ability of bathophenantroline to chelate iron. Although these differences are small, and also taking into consideration the fact that 6-sialotransferrin constitutes only 8.6 % of the total transferrin by the end of pregnancy, still this may have a profound effect on the distribution of iron over the body because the liver besides continuously taking up iron also continuously excretes iron. A small effect on the uptake may therefore have a much profounder effect on the net balance figure.
Why then would the liver behave different from the placenta and the bone marrow with respect to its iron uptake from diverse microheterogeneous transferrins ? As described in chapter 3, the predominant mode of iron uptake in the liver differs from that of placenta and bone marrow. Although the components of the system have not been identified yet, uptake of iron by the hepatocyte is consistent with release of iron from transferrin at the cell surface. Whether or not this involves reductive release is not settled, but also not relevant in this context. The main point is that since the mechanism differs from iron uptake through receptor mediated endocytosis, an alternative behaviour of transferin with respect to iron donation to hepatocytes is conceivable. If transfer of iron to bathophenantroline disulphonate by isolated rat hepatocytes indeed correlates with the activity of the transplasmalemmal NADH oxidase activity, as Sun et al maintain[154], than the following proposition may provide a biochemical mechanism operative in the distribution of iron between sites of absorption, storage and utilisation. Presently we have not yet been able to compare diferric transferrin stimulated NADH oxidase activity for the various microheterogeneous compounds, but for the moment it is inferred from the decreased transfer of iron from 5 sialo transferrin when compared to transfer from 4 sialotransferrin to bathophenantroline disulphonate by intact hepatocytes that this may lead to increased NADH levels intracellularly and acidification of the cytoplasm. This in turn may influence the mobilisation of iron from ferritin which is also NADH and pH dependent[249], thus not only decreasing iron uptake but possibly also increasing efflux of iron from the hepatocyte (figure 27). Such a link between transferrin structure and ferritin metabolism as a result of this intrinsic endocrine action of the molecule defined and modulated by the structure of glycans attached to it will be a target for future investigations.
Applying this model to pregancy, the forced mobilisation of iron from the liver can be envisioned as being due to increased concentrations of the highly sialylated transferrins that are as competent as the ordinary 4 sialotransferrin in delivering iron to bone marrow and placenta, but lag behind with respect to iron delivery to the liver. The liver efflux remaining constant or even increasing as a result of increased intracellular NADH/NAD^+ ratio, even small effects over long periods of time will lead to significant diminution of liver iron stores. It also accounts for iron taken up in hepatocytes via other routes than transferrin, which may be significant in

view of the fact that it is still unclear by which way iron taken up from the gut enters the circulation and via which route in then reaches the liver and eventually transferrin.
In iron deficiency anemia, mobilisation of iron may result from the decreased saturation of serum transferrin. In this condition (but also in pregnancy) it is noteworthy that the hepatic (reductive) release mechanism is saturated at 3μM, whereas the receptor mediated endocytosis is fully saturated at 0.3 μM. As transferrin saturation falls below 10 % in developing iron deficiency, the point is reached where diferric tetrasialotransferrin concentration becomes too low to keep hepatic transferrin receptors continually occupied, with the above indicated sequence leading to increased mobilisation of iron from ferritin as a result. Thus at low serum transferrin saturation liver uptake will be decrease more than bone marrow uptake, possibly in combination with activated mobilisation from hepatocytes. Likewise peripheral tissues may suffer from the great avidity of bone marrow for iron as a result of its specialised mode of iron uptake, and in that context the phenomenon of latent iron deficiency can be placed.

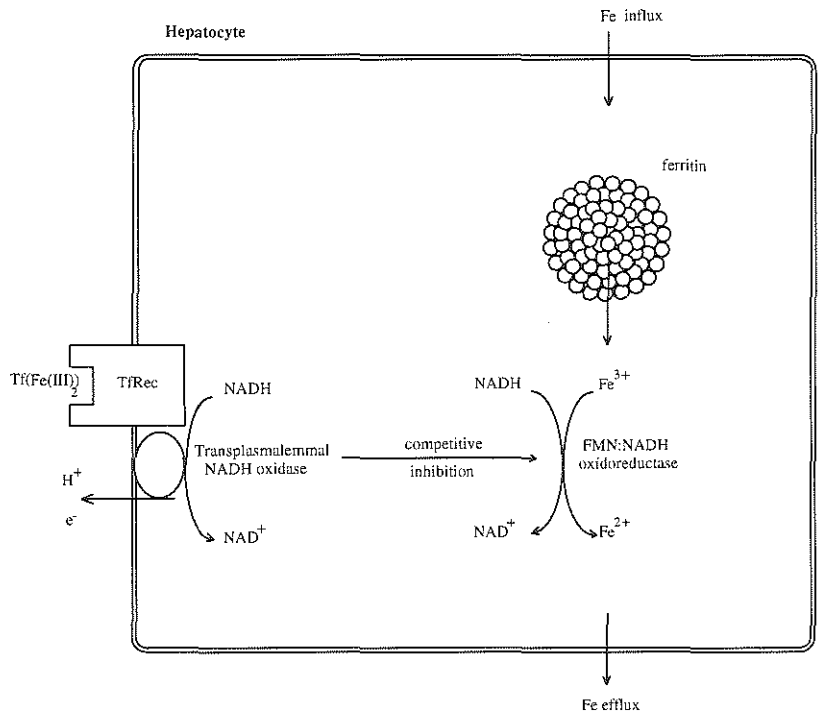


Figure 27. A hypothesis on the mobilisation of liver iron stores as a result of modification of ratios between microheterogeneous forms of transferrin. Binding of transferrin to its receptor activates the transplasmalemmal NADH oxidase. Under saturating conditions net activity will nevertheless decrease when the amount of highly sialylated transferrins increases as these activate the transplasmalemmal NADH oxidase to a lesser degree (figure 24) leading to an increased NADH/NAD^+ratio, which through its link via the FMN:NADH oxidase may lead to increased mobilisation of ferritin iron and increased iron efflux. Together with the measured decrement in iron uptake from transferrin as a result of increased glycan branching this results in both mobilisation and depletion of iron stores.

The physiological significance of the N-linked glycans, and changes observed in various conditions may be extended beyond the above described concept when other functions of transferrin are taken into consideration as well. In particular the growth stimulating effect of transferrin makes a good candidate for being susceptible to changes in microheterogeneity. Not addressed is the possibility that glycans may be important in the stabilisation of the C-terminal domain, but if present one could hardly expect microheterogeneity shifts to play any role in that field. For the moment we have identified one possible function of the N-linked glycans in iron homeostasis and hope to be able to study more structure function relationships in the near future employing the recombinant transferrins.

Summary

Combining available knowledge on the role of transferrin in iron metabolism and observations on the variability of the structure of transferrin N-linked glycans in a number of physiological and pathophysiological conditions, it is conjectured that these covalently linked oligosaccharides may play a decisive role in the perpetual redistribution of iron over sites of storage and utilisation in the body.

Additional *in vitro* experiments, assessing the interaction between transferrins distinguishable on the basis of differences in N-linked glycan structure and cells derived from its major target organs, support this notion. A possible biochemical mechanism involved in this regulating activity is pointed out as well by results obtained.

Conclusions:

1. Variation in the structure of transferrin linked glycans is a tightly governed, non random process.

2. The glycans constitute an adjustable signal attached to the protein capable of modulating iron fluxes in the body.

Samenvatting

Op grond van observaties betreffende de variatie in de structuur van aan transferrine gebonden glycanen in verscheidene physiologische en pathophysiologische condities is, vanuit de huidige stand van kennis omtrent de plaats van het transferrine in het ijzermetabolisme de hypothese opgesteld dat deze covalent aan transferrine gebonden oligosacchariden een bepalende rol zouden kunnen spelen in de uit normaal celverval voortvloeiende voortdurende herverdeling van lichaamsijzer over diens opslag- en verbruikscompartimenten.

Aanvullende *in vitro* experimenten, waarin het gedrag van verscheidene op grond van verschillen in glycaan-gedeelte gedefinieerde transferrinen ten opzichte van de belangrijkste doelorganen is geëvalueerd, verlenen steun aan deze hypothese. Daarnaast vestigen deze resultaten ook de aandacht op een biochemisch mechanisme dat aan bovengenoemde regulerende rol ten grondslag kan liggen.

Conclusies:

1. Variatie in de structuur van aan transferrine gebonden glycanen is een strikt en doelmatig gereguleerd proces.

2. In zijn structurele variatie vormt dit aan het eiwitmolecuul toegevoegde kenmerk een modificeerbaar signaal dat de verdeling van ijzer over lichaamscompartimenten kan moduleren.

Appendix A: Materials and Methods

Patients.

After acquiring informed consent, blood was obtained from 21 healthy volunteers, 8 male and 13 female, 56 pregnant women attending the University Hospital Obstetrical Clinic, 7 women using oral contraceptives, 9 women with iron deficiency anemia, and 18 female patients with rheumatoid arthritis fitting the revised ARA-criteria[250]. Anemic pregnant women and pregnant women with a latent iron deficiency were excluded by hemoglobin and serum ferritin measurements. The course of pregnancy and delivery was uncomplicated in all cases. All 18 female rheumatoid arthritis patients had active disease and used non-steroidal anti-inflammatory drugs, as well as long-acting anti-rheumatic drugs. Other causes of anemia were excluded by history taking (present or past ulcer disease or other complaints or signs of gastrointestinal diseases, hypermenorrhoea and haematuria), negative stools for occult blood, a normal vitamin B12, folic acid, Coombs test, creatinine clearance and absence of microscopic hematuria. The female rheumatoid arthritis patients were divided into three groups of six: nonanemic patients, patients with iron deficiency anemia and patients with anemia of the chronic diseases. This distinction has been made on established criteria based on hemoglobin, serum transferrin and serum ferritin measurements[235,251].

Statistical analysis.

Total transferrin concentrations were compared using the two tailed Student t-test, and non-parametric data by the Mann-Whitney U test. Coefficients of correlation were compared using Spearman's test.

Sample treatment

Venous blood samples were obtained between 10.00 and 12.00 hour in glass tubes not containing anticoagulant. Omitting the anticoagulant is a relevant part of the procedure as we and others have found that the addition may influence the isoelectric focusing[252]. After clot retraction, the sample was centrifuged for 20 minutes at 1200 g and 4°C. The serum was used immediately or stored at -20°C, or -70°C until use. Before storage or application on the gels complete iron saturation of the transferrin was ensured by mixing 100 µl serum with 5 µl of 0.5 M $NaHCO_3$ and 3 µl of 10 µM Fe(III) citrate which was then left at room temperature for 1 hour. Rabbit anti human transferrin-anti sera were home made and have been compared favourably with anti sera from Dako, Denmark.

Analytic isoelectric focusing and crossed-immunoelectrophoresis

This method involves two phases combining two electrophoretic techniques; the first establishing separation of proteins of interest, the second run perpendicularly to the first for identification and quantification purposes.

In the first phase the transferrin subfractions were separated on polyacrylamide gelstrips on an immobilized pH-gradient by isoelectric focusing. 4 mm wide strips cut from an Immobiline Dry Plate, pH 4.9-6.1 (purchased from LKB-Produkter AB, Bromma, Sweden), which had previously been rehydrated in aqua bidest to a final weight of 20 g per gel, were placed on a thermostatic template kept at 10°C. Anode and cathode strips soaked in 10 mM glutamic acid and 10 mM NaOH, respectively, were placed over the ends of the Immobiline strips. 2 µl of the iron-saturated serum was applied at the cathodal side of the strips. A voltage of 1 kV was applied during 18 h at 10°C.
For the second phase of the electrophoretic procedure, 60 ml of a 1% (w/v) solution of Agarose-M (LKB) in 24-mM sodium barbiturate-HCl buffer pH 8.7 prepared at 100°C were cooled to 55°C, whereupon a preheated (55°C) amount of the rabbit anti-human transferrin preparation was added and thoroughly mixed with the agarose solution. This mixture was poured onto a preheated glass plate (130 mm x 260 mm) placed on top of a second glass plate kept at 50°C. Then the Immobiline strips from the isoelectric focusing step, having been soaked for 15 min in the electrophoresis buffer, were placed upside down on the agarose surface. The crossed immunoelectrophoresis is carried out in the 24-mM barbiturate-HCl buffer during 4 h at 10°C at a voltage of 12-13 V/cm. All determinations were done in duplicate. The fractions were successively quantitated via measurement of the areas enclosed by the immunoelectrophoretic peaks.
Alternatively, relative amounts can be determined by immunoprecipitation of transferrin directly after ending the first phase-separation. To this end, samples must be diluted 1 : 10 before application on the immobiline strips. After the isoelectric focusing strips were covered with 50 µl rabbit anti human transferrin solution (purchased from Dako, Copenhagen, Denmark, titre 2800). Dilution of the sample is necessary as antigen excess may cause centres of the predominant subfractions to dissolve during the following wash in saline after which the gel is left in saline overnight. Bands were stained in a solution of 12 % (w / v) trichloroacetic acid containing 0.1 % Coomassie Brilliant Blue G-250, followed by staining with Coomassie Brilliant blue R-250, and finally rinsed with demineralized water and air dried.
Transferrin bands have been quantified with the Ultroscan XLaser Densitometer. Results were processed using the Gelscan XL (2.1) software (both purchased from Pharmacia / LKB).

Protein concentrations

Transferrin concentrations were measured turbidimetrically[253] using the Boehringer ES22. For this transferrin was immunoprecipitated with sheep-anti-human-transferrin (purchased from Boehringer) in an 0.1 M Na_2HPO4 / KH_2PO4 bufferr pH 7.5, containing 4% polyethyleneglycol. Ferritin concentrations were measured by ELISA.
Known transferrin functions are concentration dependent processes. Thus in all groups individual concentrations have been equated from fractional ratios and total transferrin concentration. In pregnancy and other groups this adjusts the effects to its physiological proportions, as fraction ratios (assuming unaltered, similar serum half lives) describe the relative adaptation of synthesis, but in conditions where total protein synthesis increases or decreases as well, this presentation does not correctly reflect physiological changes.

Preparative isoelectric focusing

Five cm wide strips were cut from precast Immobiline Dry Plates pH 4.9 - 6.1. Gels were rehydrated with demineralized water. Concentrated diferric transferrin C_1 (125 gram/l in 10mM $NaHCO_3$ / NaCl solution , pH 8.0) was applied directly on the gel surface 1 cm from the cathode over a width of 1 cm. A voltage of 2 kV was applied. Succesive applications of 100 µl transferrin-solution were possible when exuded droplets of transferrrin-solution were removed from their respective focusing positions twice daily. Also, excess solvent from previous applications was

removed before repeat applications using dry electrode strips and simultaneously elctrode strips were resoaked in appropriate solutions. Under these conditions focusing was allowed to continue until all of the sample had been applied and focused (in total 4 days, twice daily application of 100 µl).
Exuded droplets of transferrin solution were removed daily by absorbtion in dry pieces of electrode strip, which were then stored in closed tubes at -20°C. For recovery these electrode strips were simply eluted with a buffer solution.

Concanavalin A affinity chromatography.

Transferrin isolated from serum by immumoaffinity chromatography was separated in to the following three fractions: nonbinding, weakly binding and strongly binding following the procedure described by Hatton and Berry. Diferric C1 transferrin dialyzed against 0.05 M Tris/HCl, 1 M NaCl, 1mM $CaCl_2$, 1mM $MnCl_2$, 1mM $MgCl_2$, pH 7.6. approximately 1 mg diferric transferrin/ gram wet gel was loaded on the Sepharose concanavalin A column. The column was equilibrated with 0,05 M Tris/HCl/ 1 M NaCl, 1mM $CaCl_2$, 1mM $MnCl_2$, 1mM $MgCl_2$, pH 7.6. Fractions of 5 ml were collected. Most of the transferrin (about 76 %) was eluted only after adding α-methyl-glucopyranosyl to a concentration of 0.15 M to the equilibration buffer. The nonbound and the weakly bound fractions amounted to approximately 4 and 20 % respectively.

Differentiation of occupation of iron binding sites in serum and bone marrow.

For the analysis of iron distribution, serum or plasma, or bone marrow derived serum was diluted with an equal volume of anti-albumin antiserum raised in a rabbit by repeated intradermal injection of human albumin.
Isoelectric focusing was performed in a Phastsystem with Phastgels pH 4 - 6.5 and pH 5 - 8 (Pharmacia / LKB, Uppsala Sweden). These are polyacrylamide gels saturated with mixtures of ampholines to create the pH-gradient when subjected to an electrical field. Using immobiline derived pH-gradients has not produced reliable results with respect to the analysis of the occupation of iron binding sites, uptake of iron from these precast gels possibly providing one of the main, unavoidable obstacles.
For the analysis of iron distribution in a Phastgel IEF 5 - 8, the following program was adopted:

Sample applicator down at	step 1.2	0 Vh.
Sample applicator up at	step 1.3	0 Vh.
Separation	step 1.1	2000 V, 2.0 mA, 3.5 W, 15°C, 585 Vh.
Separation	step 1.2	200 V, 2.0 mA, 3.5 W, 15°C, 15 Vh.
Separation	step 1.3	2000 V, 2.0 mA, 3.5 W, 15°C, 450 Vh.

The four (tetra-sialo) transferrin bands that can be discerned on the basis of differences in iron content in serum and in bone marrow were quantified with the Ultroscan XLaser Densitometer. Results were processed using the Gelscan XL (2.1) software.

Amino acid analysis.

For an elaborate description see reference 254. In short, amino acids and hexosamines have been assayed in a single run on a Multichrom M Analyzer (Beckman, Münich, FRG) with a column (60 x 0.4 cm) filled with Dionex DCIA resin (Dionex Corp., Sunnyvale, CA, USA). After acid hydrolysis N-Acetyl-glucosamine was determined as glu-

cosamine. Peak areas were integrated automatically by an Infotronics CRS 210 Integrator. Sample volume 250 µl. Analyses have been performed in triplicate. Analysis of a calibration mixture with sialic acid, mannose, galactose and glucosamine added in the same composition as expected in transferrin resulted in a recovery of glucosamine of 82 % and in the presentation of results this recovery percentage has been compensated for. Values are all from 24 hour hydrolysates, thus avoiding errors introduced by extrapolations necessary to correct for hydrolysis time influences.

Carbohydrate analysis.

Hydrolysis was performed in hydrolysis tubes containing samples of 50-300 µg protein, equalling 2.5-25 nmol of individual carbohydrates, in 550 µl aqua bidest. Protein concentration was determined by measuring the E_{280}. Next followed addition of 100 µl 100% trifluoroacetic acid and the tube was degassed at -30 °C and sealed. After a 2 hour hydrolysis contents were quantitatively transferred into a tube of a vacuum rotary evaporator and evaporated to dryness at 30-50 °C, and subsequently dissolved in 100 µl aqua dest, after which 1 ml absolute ethanol was added.
Analyses have been performed on an adapted Multichrom M (Beckman, München, FRG) with a reaction coil of 5 metres and a diameter of 0.75 mm, a 30 metre teflon coil at the photometer outlet as backpressure coil to prevent air bubbles and a 60 cm long column with a diameter of 4 mm filled with Aminex A-6 resin that has been converted into its trimethylammonium form by treatment of the resin with a 2 molar trimethylammoniumchloride solution, pH = 9. The eluant composition was ethanol/water in a ratio of 89/11. Flow rates of 25 ml/h at 75 °C, or 16 ml/h at 60 °C have been applied. The eluant must be degassed before use. The colour reagant consisted of 0.02 % (w/v) p-anisyl tetrazoliumchloride in 0.18 mol/l NaOH which has to be refreshed weekly. Reagent flow 4 ml/h. The column eluate was measured at 570 nm, peak area comparison was performed with an Infotronics CRS 210 Integrator. The sample volume applied is 250 µl.
For the spectrophotometric determination of sialic acid in transferrin the optimum hydrolyzation procedure was found to be mild acid hydrolysis 0.01 M sulfuric acid at 70°C for 3 hours. Sialic acid determination was performed according to the method described by Horgan[227]

Bone marrow samples and incubation with transferrin subfractions.

Healthy donor bone marrow was obtained by aspiration from the iliac crest. Cells were immediately transferred to heparinised Hank's medium. Cells were washed in Hank's medium with 20 mM HEPES pH 7.4 before incubation and resuspended in this buffer and divided in fractions for incubation with either 4 -sialo diferric transferrin or 6-sialo diferric transferrin at a final concentration of 1 µM at 37°C under 95% oxygen and 5 % carbondioxide. Nonspecific uptake was corrected for by a simultaneous experiment with a 200 fold excess of unlabeled diferric transferrin. The experiment was performed in triplicate.
At set times samples were taken and the reaction was stopped by the addition of ice cold PBS. Cells were washed 3 times with ice cold PBS and spun down at 200 g for 10 minutes at 4 °C.
Internalized iron was assessed by gamma counting of the remaning cell pellet. Protein determination of each pellet was subsequently performed and results were normalized to counts per minute per mg protein.

Hepatocyte culture and hepatocyte incubation with transferrrin species.

HepG2 cells were grown to confluence in culture flasks. 24 hours prior to incubation experiments ^{3}H thymidine was added to the medium. Experiments were carried out in DMEM with 10 % fetal calf serum added. ^{59}Fe labelled diferric transferrins were added to the culture flasks at t = 0, to a final concentration of 0.3 µM. Nonspecific uptake was corrected for by a simultaneous experiment with a 200 fold excess of unlabeled diferric transferrin. Asialoglycoprotein receptor mediated uptake was detremined by simultaneous experiments with additional asialofetuin. At set times flasks were put on ice and rinsed with ice cold PBS 3 times. Then cells were harvested after a 5 minute trypsin incubation and washed again with cold PBS. Iron uptake was determined from the gamma counter and normalization of values for differences in pellet size were performed on the basis of β-scintillation from incorporated ^{3}H thymidine.
Rat hepatocytes were isolated as described below and incubated with ^{59}Fe labelled diferric 4 and 6 sialotransferrins in PBS pH=7.4 for two hours at a final concentration of 5 µM at 37°C under 95% oxygen and 5 % carbondioxide. Nonspecific uptake was corrected for by a simultaneous experiment with a 200 fold excess of unlabeled diferric transferrin. Asialoglycoprotein receptor mediated uptake was detremined by simultaneous experiments with additional asialofetuin. At set times aliquots were drawn, then centrifuged at 2000 g for 15 minutes. Cells were resuspended and washed briefly in ice cooled PBS and centrifuged. This was repeated once again after which cells were finally pelleted abd separated from supernatant for γ-conting. Total pellet protein was determined in all pellets separately after this and results wre normalized for these values.

Preparation of placental brush border vesicles

Full term human placenta was put on ice and transported to the lab in 20 - 30 minutes after delivery. The isolation of microvillous membrane is based on the method of Smith et al[255] The placenta is rinsed with ice cold PBS. Villous tissue fragments of 5 - 10 mm size are cut from the maternal side of the placenta, rinsed in PBS and then left in 5 % CO_2 / 95 % O_2 - gassed Hanks, pH 7.4. Usual harvest produced 70 - 120 grams of placental villous tissue.
This tissue was rinsed 3 times with 0.1 M $CaCl_2$, then 3 times in PBS and finally resuspended in 150 ml PBS. All solutions kept at 4 °C. Using a magnetic sirrer, the solutions is stirred for 30 minutes, large tissue fragments are disposed of.
Next the suspension is filtered through nylon mesh (pore-size 90 µm). The filtrate is kept and centrifuged at 800 g for 10 minutes. Supernatant is separated from pellet and the former is centrifuged at 10,400 g for 30 minutes (60 Ti Rotor 10,500 rpm, brakes off). Pellet and supernatant are separated and supernatant is centrifuged at 110,000 g for 30 minutes (50.2 Ti rotor 31,000 rpm, brakes off). Supernatant is discarded and pellet is suspended in PBS. A Potter-Elvehjem homogeniser (40 sec at 25 rpm, clearance 0.004 - 0.006 inch) was used in all resuspension steps.
Choatropous treatment consisted of resuspension of pellet in 0.2 M NH_4SCN, incubation in this medium for 10 minutes, followed by pelleting membrane vesicles by centrifugation at 110,000 g for 30 minutes. This step was repeated once, after this pellet was resuspended and homogenized in PBS and used directly in assays or kept at -20 °C until use.

Transferring binding studies

Purified diferric transferrin subfractions were ^{125}I-labeld employing the Iodogen-method. After reacting preparation

were immediately loaded on a Sephadexx G 100 column and eluted with PBS pH=7.4. Eluant was collected in fractions that were tested for radioactivity and transferrin containing fractions were subsequently transferred into dialysis membranes. Dialysis against 3 changes PBS pH=7.4 for 24 hours followed to ensure optimal removal of unbound iodine. Specific activities obtained ranged from 800-1000 cpm/nanogram Tf.

For the binding assays stored suspended frozen placental membrane vesicles were thawed, stirrred and then pelleted by centrifugation at 110,000 g for 30 minutes (50.2 Ti rotor 31,000 rpm, brakes off). Supernatant was discarded and pellet suspended in PBS. For each sample resuspended solution to a volume containing 100 µg membrane vesicle protein was added, and total endvolume was 230 µl. After addition of labeled transferrin the mixture was left at 22°C for 1 hour, followed by centrifugation at 110,000 g for 30 minutes. Supernatant was spearated from pellet, and the pllet rinsed 3 times with PBS pH 7.4 , supernatant from rinses was added to inital supernatant. γ-Counting of pellets and supernatants provided data for calculation of bound and free transferrin. Data for bound transferrin were all normalized to pellet samples 100 µg membrane vesicle protein by protein determination of all separate samples. Nonlinear regression analyis of data was performed on the basis of these data using the software contained in the SPSS-program (Marquardt-Leventhal algorithm). Results in table are means from three separate experiments.

Indirect assesment of NADH-oxidase activity of transferrin species

Hepatocytes were isolated from male Wistar rat by the method of Berry and Friend[257]. Cells were finally supended in buffer containing 50 mM HEPES, 110 mM NaCl, 5 mM KCl, 0.74 mM $MgCl_2$, 1,26 mM $CaCl_2$, 1% BSA, pH 7.4. $5 \cdot 10^6$ cells /ml were incubated at 37 °C with 1 mM batophenantroline in the absence or presence of either 4-sialo or 5-sialo diferric transferrin at a concentration of 5 µM. Aliquots of cell suspension were taken at set times and centrifuged in an Eppendorf centrifuge 5414 for 15 seconds. The absorbance of the clear supernatant at 535 nm was then determined. Results from three consequtive experiments are shown.

The millimolar extinction coefficient of the iron-bathophenantroline complex at 535 nm used in equations was 22.14 $cm^{-1}mM^{-1}$.

Equilibrium dialysis

Transferrin subspecies isolated by isoelectric focusing were dialyzed against 2 changes of EDTA, 3 changes of demineralized water and two changes PBS. Dialysis membranes were filled with determined quantities of respective sialotransferrin variants and then dialyzed against a ferric iron solutions for 5 days. Concentration of protein and iron in the dialysis cell and in the dialysate were determined and affinity constants determined according to[244].

Isolation of recombinant human transferrin from E. coli

A 25 ml sample of E.coli (LC 137) in LC medium was grown overnight in an airshaker at 37 °C. After 22 hours this culture was added to 1 liter of LC medium containig 0.84 mM IPTG. Cells were grown overnight and harvested in an MSE Coolspin centrifuge at 2600 g for 15 minutes. Cells were resuspended in 10 mls 50mM Tris/HCl, 1 mM EDTA, 250mM NaCl, 5 mM DTT, pH 8.0 and disrupted by adding 100,000 U lysosyme.after a 30 minute incubation this mixture was snap frozen in carbice cooled ethanol. The thawed sample was sonificated, 300 U DNAse was added and the sample was incubated for 45 minutes at room temperature.

Inclusion bodies were isolated from the cell lysate by centrifugation at 12,000 g for 15 minutes. after 3 wahes in 50 mM Tris?HCl, 1 mM DTT, pH 8.0 inclusion bodies were dissolved in 10 ml 8 M ureum, 1 mM DTT, 40 mM

Tris/HCl, 10 % glycerol (v/v), pH 7.6.
Preparative SDS electrophoresis was used to isolate recombinant transferrins fro bacterial components. This was performed with a model 392 BioRad Prep Cell.

Renaturation of recombinant human transferrrin

Purified recombinant human transferrin was diluted with thoroughly degassed elution buffer to a final concentration of approximately 5 µg/ml. 0.1 MM EDTA, 40 mM KCl, 5 mM DTT and 1 mM GSH were added and pH was adjusted to 8.2. Incubation at 37 °C for 30 minutes followed and the solution was subsequently kept at 0 °C for 50 minutes. After this, 0.5 mM GSSG was added and the solution was placed at 22 °C for four hours. The renaturation was performed under an atmosphere of nitrogen.

Appendix B: Addendum results

Table 8'. Standard deviations of proportional distribution of transferrin subfractions in groups as defined in table 6.

Group	mono	di	tri	tetra	penta	hexa	hepta
M	0.0	0.2	0.8	1.6	1.1	0.7	0.0
C	0.0	0.4	1.0	2.2	1.2	0.7	0.0
I	0.0	0.8	1.7	1.6	1.9	0.9	0.1
II	0.0	0.6	1.4	1.8	1.4	0.4	0.4
III	0.0	0.7	1.9	3.4	1.6	0.9	0.9
AT	0.0	0.3	0.9	3.0	1.2	0.5	0.5
PP	0.0	0.8	1.1	2.2	1.2	0.3	0.2
PPM	0.0	0.4	1.7	1.8	1.1	0.1	0.1
Fe	0.1	0.6	1.1	3.2	2.2	1.1	0.4
OAC	0.0	0.2	0.7	1.2	0.8	0.6	0.2
RA-NA	0.0	0.5	1.4	1.5	2.3	0.5	0.2
RA-Fe	0.0	0.5	1.3	3.3	2.8	2.0	0.4
RA-ACD	0.0	0.3	1.0	3.3	1.7	1.7	0.4

Table 9'. Standard deviations of concentrations of transferrin subfractions in groups as defined in table 6. Results in µmol/l.

Group	di	tri	tetra	penta	hexa	hepta	Total
M	0.3	0.5	2.9	1.2	0.4	0.0	4.9
C	0.2	0.4	1.7	0.7	0.3	0.0	2.4
I	0.3	0.6	2.8	1.0	0.5	0.0	3.9
II	0.5	0.8	5.3	2.2	1.2	0.2	9.4
III	0.5	1.2	3.1	2.3	1.6	0.6	7.6
AT	0.1	0.4	4.5	1.4	0.7	0.3	5.7
PP	0.6	0.9	3.5	1.9	0.8	0.2	7.5
PPM	0.2	0.9	2.4	1.9	0.3	0.0	3.6
Fe	0.2	0.8	2.1	1.8	0.7	0.2	5.9
OAC	0.1	0.4	2.8	1.2	0.3	0.2	4.8
RA-NA	0.2	0.7	4.8	1.7	0.5	0.1	7.4
RA-Fe	0.2	0.7	4.0	0.9	0.4	0.1	4.9
RA-ACD	0.1	0.3	2.8	1.2	0.5	0.1	4.5

Table 11'. Standard deviations of iron uptake from transferrin by HepG2 cells (pmol Fe / 10^6 cells / hr).

Transferrin fraction	total uptake	Tf R-mediated uptake	ASGPR-mediated uptake
2 Sialo-transferrin	0.12	0.08	0.07
4 Sialo-transferrin	0.13	0.12	0.08
5 Sialo-transferrin	0.12	0.12	0.06
6 Sialo-transferrin	0.11	0.11	0.03

Table 12'. Standard deviations of iron uptake from transferrin by rat hepatocytes (pmol Fe / 10^6 cells / hr).

Transferrin fraction	total uptake	Tf R-mediated uptake	ASGPR-mediated uptake
4 Sialo-transferrin	0.08	0.09	0.03
6 Sialo-transferrin	0.07	0.09	0.02

Appendix C: References

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Curriculum Vitae

De schrijver van dit proefschrift is op 29 mei 1960 geboren te Rotterdam, behaalde in 1978 zijn VWO diploma aan de Melanchton scholengemeenschap te Rotterdam, en studeerde van october 1980 tot mei 1987 Geneeskunde aan de Erasmus Universiteit, eveneens ter stede.
Van april 1987 tot april 1989 was hij aangesteld als wetenschappelijk medewerker bij de afdeling Chemische Pathologie van de Erasmus Universiteit Rotterdam werd een aanvang gemaakt met het in dit proefschrift beschreven werk.
Sedert april 1989 is hij werkzaam bij de afdeling Interne Geneeskunde II, in opleiding tot internist.
Onderbrekingen in dit geografisch monotone bestaan vormden het keuzeprakticum van februari 1983 tot en met juli 1983 (Onderzoek betreffende in situ hybridisatie-technieken aan het Queen Elizabeth College, London, UK), keuzecoschappen Gastroenterologie en Anaesthesie van october 1985 tot en met februari 1986 in respectievelijk Syracuse NY en San Antonio TX, USA, en de stages Endocrinology (Professor G.M.B. Besser) en Medical Oncology (Prof J. Malpas) van januari 1991 tot februari 1992 in het St. Bartholomew's Hospital en Homerton Hospital te London, United Kingdom.

Naschrift

Velen hebben bewust of onbewust een bijdrage geleverd in de totstandkoming van dit proefschrift. Allen mijn dank.
Speciale vermelding is wel op zijn plaats voor een aantal van degenen die zich in de afgelopen jaren bewust voor dit onderzoek hebben ingezet. In de allereerste plaats gaat mijn dank uit naar mijn promotor.
Hooggeleerde heer, beste Henk, de eerste aanzet tot dit onderzoek is van jou gekomen en ik heb mij erin kunnen verheugen dat jij mij dit onderwerp hebt willen toevertrouwen. Daarbij genomen de onafgebroken ondersteuning van mijn onderzoeksinitiatieven en de in jou gewaardeerde neiging om naast een waar promotor ook een mentor voor jouw promovendi te zijn, heeft de samenwerking van een bijzondere kwaliteit mogen doen zijn.
Wim van Noort wil ik hierbij ook in het voetlicht zetten. Menige discussie hebben wij gevoerd waarbij ik vooral jouw als analist door de praktijk gevormde invalshoek en inzicht steeds hoog gewaardeerd heb. Samen met de vanuit intellectuele hoek insprirerende discussies met Hans van Dijk hebben deze gemaakt dat ik uiteindelijk toch een inzicht in de materie heb kunnen verwerven die althans mijzelf tot tevredenheid kon stemmen.
Nel de Jeu en Martin Kroos wil ik ook apart noemen, met dank voor hun uitstekende werk op het laboratorium van de afdeling Chemische Pathologie.
Maarten de Smit heeft onze afdeling een grote dienst bewezen middels het tot expressie brengen van het humane transferrine gen in de E. coli, en wij hopen hiervan in de komende jaren de wetenschappelijke vruchten van te kunnen plukken.
Paul Wilson dank ik voor zijn ondersteuning van mijn voornemen een extra wetenschappelijke stage in de opleiding in te plannen teneinde het laatste practische werk te kunnen uitvoeren, en ook voor zijn spoedige beoordeling van het inderhaast geschreven manuscript, hetgeen eveneens moge gelden voor Professor Koster en Professor van Dam.
Rest mij nog Harrie te danken voor zijn hulp, meer dan eens tot in de nachtelijke uren, bij de daadwerkelijke totstandkoming van het drukwerk.

Masha, Thomas, Mark en Iris zijn in de afgelopen jaren, vooral dankzij de liefhebbende zorg van Debora weinig tot last en tot veel levenslust geweest. Zelfs het jaar in Engeland zijn jullie allen steeds dicht bij mij geweest, een ware familie.