THE INTERACTION OF RAT TRANSFERRIN WITH THE LIVER WITH SPECIAL REFERENCE TO THE GLYCAN OF TRANSFERRIN

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THE INTERACTION OF RAT TRANSFERRIN WITH THE LIVER

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(ABSTRACT)

Rat transferrin (RTf) was used to study the interaction of transferrin (Tf) with the liver, with special reference to the glycan of Tf.

Iron uptake from Tf by cells is believed to occur by Tf receptor (TfR)-mediated endocytosis. Results from the present studies revealed that over a large range of competing diferric RTf concentrations, a constant percentage of iron is uptaken from RTf by the liver in vivo, and by hepatocytes in vitro. Hepatocytes were found to contain ~129,000 TfR/cell with ~40% expressed on the surface as estimated by both a polyclonal anti-TfR antiserum, produced as part of this thesis work, and a monoclonal anti-TfR antibody. On the basis of this estimate it was calculated that TfRs are not present in sufficient quantities to account for the observed uptake of iron. Studies of uptake and release of polyvinyl-pyrrolidone (PVP), RTf, and iron (as diferric Tf) were carried out in suspended hepatocytes. These studies demonstrated that the most likely mechanism to account for the results and to explain the iron uptake is "mixed-type" pinocytosis. The uptake of iron was found to be modulated by the type of glycan on RTf.

On the basis of glycan microheterogeneity, at least six subforms of RTf are found to exist in rat plasma. These subforms are RTf-1, RTf-2 and RTf-3 as

resolved by concanavalin A; ~20% of each is fucosylated and ~80% non-fucosylated. (The presence of fucose was found to have no measurable effect on catabolic rate, plasma iron disappearance or iron donation to liver *in vivo* or hepatocytes *in vitro*.) The sialylated subforms have different half-lives (RTf-1>RTf-2>RTf-3) with RTf-1 being significantly longer than RTf-3. Comparison of plasma iron disappearance and rates of iron donation to liver *in vivo* suggested a trend (RTf-1>RTf-3>RTf-2) which was reproduced and found to be significant in studies with hepatocytes: iron uptake by hepatocytes from RTf-1 and RTf-3 could be competitively inhibited by an excess of the homologous subform. Desialylation of the subforms (RAsTfs) significantly reduced the half-lives and altered the order (RAsTf-3>RAsTf-1>RAsTf-2) with RAsTf-3 being significantly longer than RAsTf-2. The desialylated subforms were superior donors of iron to the liver *in vivo*. Studies to explain the enhanced rate of iron delivery by RAsTf, discounted the possibility of differing rates of iron release, but allowed postulation of a synergistic dual receptor mechanism. Results from experiments with hepatocytes *in vitro* supported the proposed mechanism. It is concluded that subtle differences in glycan structure can result in functional differences between Tf subforms.

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CHAPTER 1

INTRODUCTION TO TRANSFERRIN

INGENIOUS are the mechanisms and systems which nature has designed on her evolution drawing board in order to maintain a dynamic physiological homeostasis within the living organism.

Harnessing energy and putting it to useful purposes involved the shuttling of electrons through the biosystem; a capable and available candidate for the job was the iron atom. In the beginning, the atmosphere was essentially oxygen free and iron was able to exist in its ferrous state in solution. But, with the coming of oxygen synthesizing organisms, the oxygen atmosphere resulted in the oxidation of ferrous into ferric iron in which at neutral pH in aqueous solution binds tightly to a first molecule of water. Accompanying this is a reduced pKa and the release of a proton bringing about the availability of a hydroxyl ligand which has affinity for a second ferric ion, and so on. Ultimately this leads to polymer formation and precipitation out of solution. Clearly this is an undesirable situation for the living organism. Thus a mechanism for delivery and keeping Fe^{3+} in solution was evolved.

The presence of low weight compounds which are able to chelate ferric iron, such as citrate, had probably existed long before the advent of the protein carrier. But as organisms became multi-cellular, the ability to regulate the flux of iron both into and out of the intracellular milieu necessitated a system which allowed partitioning of iron between the intra- and extracellular spaces. The low weight chelators would not be as effectively excluded by the cellular plasma membrane as would a protein of greater molecular weight. Based on the physical characteristics of the present-day iron carrying proteins, it has been postulated that the earliest form of such proteins probably only carried one iron atom and had a molecular weight of approximately 40 000 daltons (Da)(Aisen and Listowsky, 1980). More recently, Martin et al. (1983) have been able to identify an iron carrying molecule in a urochordate Pyura stolonifera which, indeed, contains one binding site for iron and has a molecular weight of approximately 40 000 Da.

It has been known since 1898 that iron exists in our blood plasma and it has been known since 1927 that it is bound to a protein (Weinberg, 1984). However, it was not until 1946 when this iron binding protein was first isolated and called siderophilin - meaning iron-loving

(Schade and Caroline, 1946). In a report the following year, Holmberg and Laurell (1947) coined its present day appellation: transferrin. The term was meant to signify its whole body role of TRANSferring iron throughout the circulations. History has provided a plethora of terms for this molecule. Thus some of the other names given to transferrin are included here to help avoid confusion for the reader of literature on this subject. It has been variously called, serum transferrin, sero transferrin, iron-binding protein, β_1-metal-binding protein and as mentioned above siderophilin. It is not to be confused with ovotransferrin which is the chicken's counterpart or lactoferrin which is a distinctly different iron-binding protein commonly found in milk and other secretions such as tears (Aisen, 1980).

There is significant polymorphism in transferrin expression and it was first described by Smithes (1957) when he subjected human plasma to starch gel electrophoresis. The type found to occur with the greatest frequency throughout the world population is called transferrin C (TfC). However there exists types TfC-1 through TfC-15 with the types TfC-5 to TfC-15 occurring rarely and demonstrating restricted ethnic and geographic distribution (Kamboh and Ferrell, 1987). In addition there are other types which have been described and

include possibly six different Tf D types and three Tf B variants (Yuasa et al., 1987). The reason for these various forms of expression probably relates to the geographic and ethnic distribution where depending upon the particular evolutionary pressures exerted, subtle alterations in the molecule enhanced or detracted from its role as a growth factor, bacterio-stat or immune system actor as was needed.

1.1 Physical Characteristics

Early ultracentrifugal studies of human, monkey and rat transferrins gave estimates of their weight at approximately 68 000 D (Charlwood, 1963). However this estimate has been revised [1-1]* and is now established at 79 550 Da.

Transferrin is composed of two domains, within each of which approximately 40% of the amino acid residues are homologous[1-2] (Baldwin and Egan, 1987) (see Figure 1.1).

In its tertiary configuration and when it is charged with two atoms of iron, transferrin resembles a prolate ellipsoid of revolution with a ratio of major to minor axes equal to 2.1 (Rossneu-Moutreff et al., 1971).

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*This note and subsequent notes in this chapter are located in Appendix CH-1.

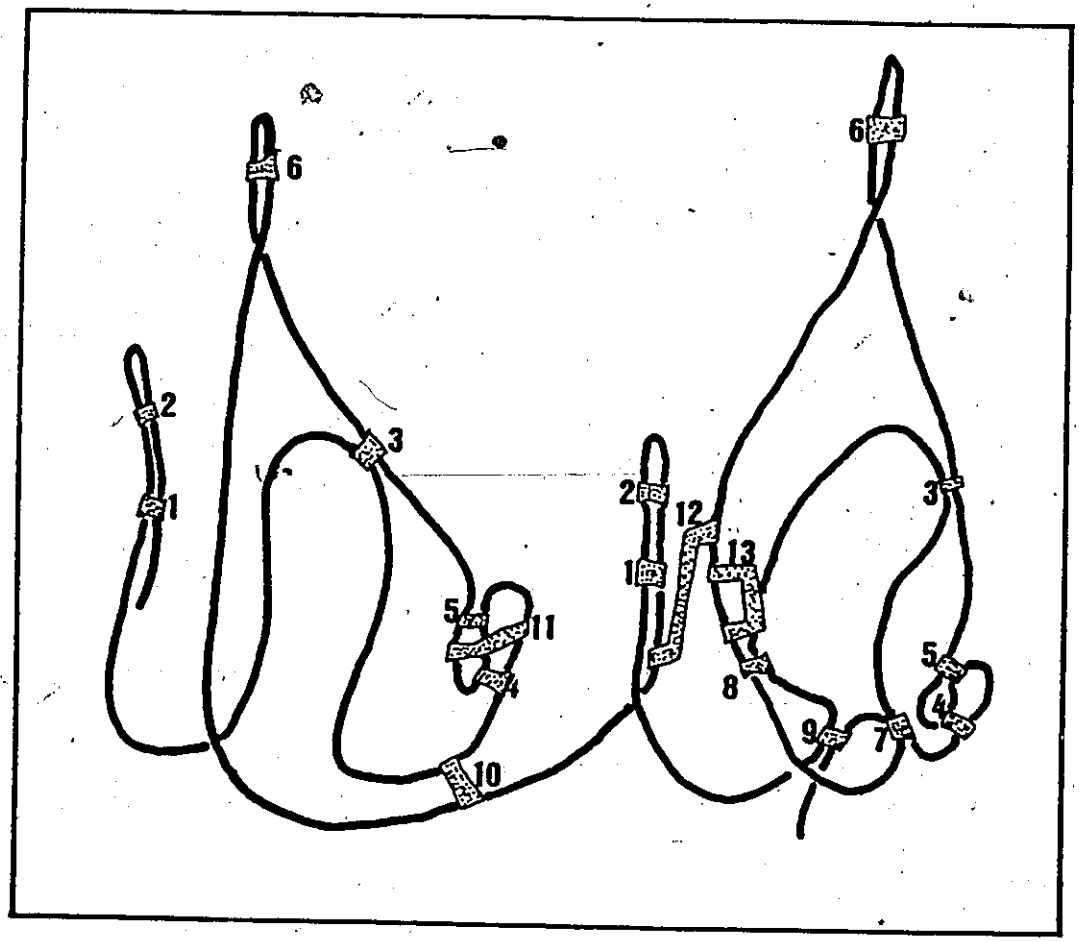


FIGURE 1.1

FIGURE 1.1 A string model demonstrating the similarity of domains and the disulphide bridges in human transferrin. The N-domain on the left.
(After Williams, 1982)

Meares (1982) conducted a study of the iron locations in transferrin by fluoresence energy transfer and estimated that the sites were each approximately 10.6 ± 0.4 Å below the surface of the protein; the intersite distance is on the order of 35.5 ± 4.5 Å (O'Hara et al., 1981).

1.2 Coordination of Iron

When two atoms of iron are complexed to transferrin the resulting diferric transferrin becomes characteristically salmon-pink coloured (Schade and Caroline, 1946). Associated with this phenomenon is an increase in absorbance of light energy at a wavelength of 470 nm (Aasa et al., 1963). A useful feature which arises from this finding is that the ratio of 465/280 nm absorbances for diferric transferrin yields a constant equal to 0.046.

In addition to binding iron, a few other metals of physiological interest have been found to be chelated by transferrin and they include[1-3]: copper, gallium, chromium, cobalt, manganese, vanadium, aluminum and zinc. However, the primary candidate for site occupancy, by at least an order of magnitude, is iron.

Ferric iron (Fe^{3+}) is most commonly arranged in a six-coordinate octahedral geometry[1-4] and enters into both Pi and sigma bonding arrangements with biomolecules

(Bates, 1982). Two of the sites of coordination are with ligands extrinsic to the transferrin molecule. One of these sites is occupied by a bicarbonate or carbonate anion[1-5] while the other[1-6] is a water or hydroxide molecule. Concerning the remaining four sites of coordination and stabilization of the anion, Figure 1.2 depicts the most probable amino acid sites of interaction[1-7] between the atom of iron and the amino acids, and the bicarbonate/carbonate anion on the transferrin molecule (Chasteen, 1983).

The likely sequence of events surrounding the complexing of Fe^{3+} to a transferrin binding site begins with reaction of the bicarbonate/carbonate anion and arginine (see inset Figure 1.2)(Bates and Schlabach, 1975). This is probably followed by binding of Fe^{3+} to the two tyrosines and the bicarbonate/carbonate all of which would induce subtle folding of the protein and subsequent binding of two histidines (Chasteen, 1983). Given sufficient iron these events would be repeated similarly and almost simultaneously at the other site.[1-8]

The affinities of the two sites, which would be expressly relevant during iron loading, have been found to differ with the C-terminal site expressing a higher association constant: compare $1.0 \times 10^{22.8} M^{-1}$ with $1.0 \times$

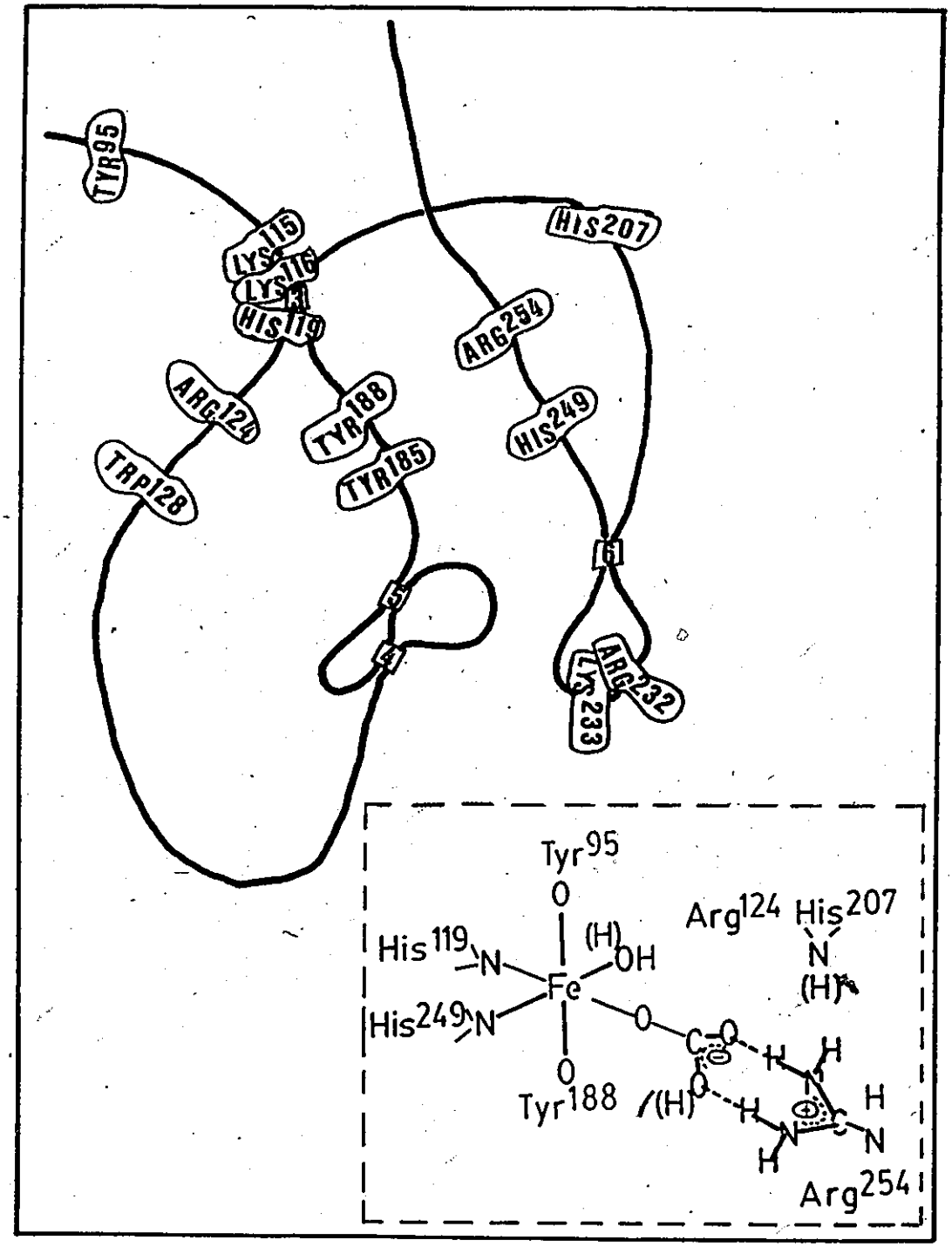

TYR95
LYS115
LYS116
HIS119
ARG124
TRP128
TYR188
TYR185
HIS207
ARG254
HIS249
ARG232
LYS233
Tyr95
His119
His249
Fe
Tyr188
Arg124
His207
Arg254

FIGURE 1.2

FIGURE 1.2 Proposed iron and anion binding peptide of the N-terminal domain of human transferrin. The probable iron ligands are Tyr-185, Tyr-188 and two of three histidines: His-119, His-207 and His-249. Arg-124 is a strong candidate for the site of anion binding, however the other cationic residues cannot be excluded. Lysine residues which probably influence the properties of the metal centre are illustrated. Disulphide bonds correspond to those depicted in Figure 1.1. (After Chasteen, 1983)

INSET A suggested structure for the N-terminal Fe^{3+} binding site for human transferrin. The choice of Arg-254 rather than Arg-124 as binding to bicarbonate is arbitrary. (H) reflects uncertainty about the location of the proton. (The corresponding C-terminal residues are listed in Table 1 of note 1.7 in Appendix CH-1) (After Baldwin and Egan, 1987)

$10^{21.5}$ M^{-1} for the N-terminal site at pH 7.4 and a bicarbonate concentration equal to 27 mM (Harris and Pecoraro, 1983). With repect to release, the ability of anions in solution to destabilize the complex (Penner et al., 1987; Folajtar and Chasteen, 1982) has been exploited to demonstrate that iron is released at different rates compared to each other in vitro. In experiments with 2,3 diphosphoglycerate as the destabilizing anion and at pH 7.1, Morgan et al. (1978) showed that in human transferrin the N-terminal site was freed of iron with a half-life of approximately 5 min while the C- terminal yielded its iron with a half-life of approximately 38 min. Qualitatively similar results were obtained in similar experiments with rat transferrin conducted at pH 6.9.

Varying the prevailing pH of a solution containing diferric transferrin reveals another aspect of the difference in the stability of the A (or C-terminal site) and B (or N-terminal site) site Fe^{3+}- transferrin-CO_3^{2-} complexes.[1-3] The A site has been demonstrated to lose its atom of iron at a pH over a range of 5.7 - 6.0 (Lestas, 1976) while the B site loses its iron at approximately pH 4.8. This aspect of transferrin appears to feature high in the cellular mechanism of iron uptake from transferrin (see below).

It has been established (Morgan et al., 1978; Baldwin et al., 1982) that the rate of release for each site is independent of the occupancy at the other site, i.e., the sites exhibit non-cooperativity. Further, it appears that in the absence of chelators, there is little, if any, exchange of iron between sites. Even rat transferrin incubated at 37°C in rat plasma apparently suffers little or no redistribution of iron between sites (Huebers et al., 1981b). There is, however, exchange and redistribution in the presence of chelators and/or reduced pH (Fletcher and Huehns, 1968; Aisen and Leibman, 1968; Lestas, 1976).

The differences in physical characteristics reported to exist between the two iron-binding sites of transferrin inevitably lead to experiments designed to investigate whether this difference translated into observable phenomena <u>in vivo</u>, or at least with living tissue <u>in vitro</u>. In 1967 Fletcher and Huehns hypothesized[1-10] that there were physiological differences and that indeed, this may relate to tissue selective site delivery of iron. However, since that time, the weight of the evidence is in favour of the view that there is no difference between the sites with respect to delivery of iron to tissues, although the issue is not completely resolved.[1-11] It is clear however that the efficiency of

iron delivery to tissues by transferrin is highly correlated to the degree of site occupancy. Jandl and Katz (1963) first demonstrated that iron is most rapidly uptaken by immature erythroid cells from rabbit diferric transferrin and most recently this observation has been quantified (8:1 to 14:1; diferric:monoferric molecular advantage) and extended and shown to be true for rat, human, baboon, and dog transferrins as well (Huebers et al., 1985). In fact, it is this advantage which diferric transferin has for uptake by reticulocytes (wherein both atoms of iron are released (Huebers et al., 1978; Morgan et al., 1967) which probably accounts for Fletcher and Huehns (1967) findings (Huebers and Finch, 1987).

Transferrin is a glycoprotein and inspite of the presence of two glycan structures (in human only, see below in section 1.3.3) on each molecule there is surprisingly little information about the possible interaction between these carbohydrate chains and the association/dissociation of iron. There is some suggestion that the glycan is more accessible to concon-avalin-A when the protein is in the apo state (Hatton and Berry, 1985) but no information relates to interactions with iron binding/release. Considering this deficiency of information rat transferrin was used as a model of glycosylated transferrin (for reasons in detail see below

and Chapter 2) and an examination of possible interaction was carried out as one of the objectives of this work (see Chapter 4).

1.3 Synthesis of Transferrin

Most work on the synthesis of transferrin has been performed in non-human systems. Morgan and Peters Jr. (1985) have shown that formation of the primary amino acid chain occurs in the rough endoplasmic reticulum (RER) and that all of the intrachain disulphide bonding occurs within 1 to 2 minutes of chain completion. They found that the conformation obtained, even at this early stage, conferred the normal iron coordinating and donating capacities on the transferrin molecule.

1.3.1 Sites of Synthesis

Total protein, including transferrin, synthesis is greatest in the rat liver at 6 months of age, it declines to a low at 24 months, then is followed by a rise between 24 to 30 months (Bolla and Greenblatt, 1982). As intimated, the principle site of transferrin synthesis is the liver displaying the highest levels of transferrin messenger RNA (mRNA) (Aldred et al., 1987) with approximately 6,500 molecules/cell (by inference in parenchymal cells, however, the distribution among cell

types was not stated) (Idzerda et al., 1986). Two other major sites of transferrin synthesis which have only recently received attention are: the brain[1-12] (Connor and Fine, 1986) and the testis[1-13] (Bridges et al., 1986). The presence of these three sites of transferrin synthesis underlines the importance of this protein in overall iron delivery. Both the blood-brain and testis barriers are effective at eliminating virtually all macromolecular exchange between themselves and the general circulation. Yet both systems clearly require a supply of iron for their respective tissues. Consequently the ability to produce transferrin independently[1-14] within each of these circulations has evolved. (Huebers and Finch, 1987).

Other sites of transferrin synthesis which have been identified include: 1) the stomach[1-15] (Baldwin et al., 1986; Aldred et al., 1987); 2) Recently Lum et al. (1986) have found by examining peripheral blood for transferrin mRNA transcription that T4 inducer T-lymphocytes are capable of synthesizing transferrin; 3) the mammary gland[1-16]; 4) the spleen (Bolla and Greenblatt, 1982; Aldred et al., 1987); 5) bone marrow (Bolla and Greenblatt, 1982); and 6) kidney, muscle and heart (Bomford and Munro, 1985; Aldred et al., 1987). On

the basis of detectable mRNA, Aldred et al. (1987) found that yolk sac, adrenals, and small intestine do not synthesize transferrin.

It has been estimated that in total, on average, approximately 1 g of transferrin is synthesized[1-17] each day in man (Bomford and Munro, 1985).

1.3.2 Factors Influencing Synthesis

The transferrin gene has been localized to chromosome number three in man (Young et al., 1984) and the mRNA produced by the gene is approximately 2 400 to 2 500 bases long (Uzan et al., 1984). There is some evidence to suggest that it is regulated by the amount of cellular iron.[1-18]

Other factors may have greater influence over transferrin synthesis in the testis. Djakiew et al. (1986) have reported that follicle stimulating hormone (FSH) and testosterone increase secretion of transferrin by Sertoli cells and Perez-Infante et al. (1986) confirmed this finding with testosterone but also found that insulin and epidermal growth factor (EGF) had a similar positive effect. In another report, the transferrin mRNA levels of Sertoli cells were also found to increase by addition of vitamin A (Huggenvik et al., 1987). With respect to synthesis in lymphocytes, it

appears as though a factor important in inflammation, interleukin-2 (IL-2), and its receptor must be expressed before there is transferrin mRNA transcription and co-expression of the transferrin receptor (Lum et al., 1986).

To date, studies of factors affecting the synthesis of transferrin at other tissue sites are lacking. Given the findings thus far however, it is possible to piece together a scenario of cohesiveness concerning the effect of the factors described above and their physiological relevance. If the primary role of transferrin is to sequester iron and shuttle it from tissue site to tissue site then maintaining an adequate level of transferrin in the circulation to perform this function is obviously important. Thus one would expect the site of serum transferrin synthesis (i.e. liver) to respond to varying levels of whole body iron. Correspondingly, if iron is needed by the body and transferrin does indeed play a role in its absorption from the gut then an increase in synthesis (or Tf's presence in the gut via the bile) would be expected in iron deficiency. Transferrin in the testis appears to play the role of a growth factor more than anything else (see discussion below) and thus it would be expected to respond to stimuli which enhance tissue growth such as EGF and testosterone. The same argument may apply to the brain

circulation, however it seems more likely that iron content would be more of an important regulating factor especially in view of the critical importance of respiration pathways to the well functioning of this organ. In the periphery, local sites of tissue injury or infection resulting in the inflammatory process could cause synthesis of transferrin by lymphocytes; with it more transferrin would be present locally and there would be enhanced sequestration of available iron. This iron would then be effectively withheld from invading bacteria (Weinberg, 1984). The coincident synthesis of transferrin receptors in these cells would increase the number of transferrin-to-cell cycles which could occur, increasing local storage of iron and allowing each transferrin molecule to capture many atoms of iron. Indeed, iron uptake and storage in peripheral blood lymphocytes has been demonstrated (Summers and Jacob, 1976).

1.3.3 Glycosylation

Depending on the protein, coincident with or very soon after synthesis of the amino acid backbone, a dolichol phosphate-linked precursor oligosaccharide is transferred to the polypeptide (Berger et al., 1982). In human transferrin this occurs at two locations:

asparagines 413 (site 1) and 611 (site 2) of the C-terminal domain. The rest of mammalian transferrins studied thus far have been found to be glycosylated only at a single site in the C-terminal domain, at as yet undetermined positions. Still within the endoplasmic reticulum (ER), minimal, preliminary trimming of saccharides on the glycan occurs (Spiro and Spiro, 1982) resulting in $Man_9(\bullet)GlcNAc_2$-N-transferrin (see Figure 1.3 (1)). After passage through the Golgi[1-19] the carbohydrate chains of all transferrins (discovered thus far) end up in the form of an N-linked complex glycan structure (see Figure 1.3, (2) for examples).

The existence of microheterogeneity (structural differences between the glycans of homologous glycoproteins) within human transferrin was first recognized by Spik et al. (1974) and was further emphasized by the demonstration of three peaks (A,B and C) within the chromatographic profile of transferrin type C on diethylaminoethyl-cellulose (DEAE) (Regoeczi et al.,1977). It was subsequently found that peak B is almost completely bound by Sepharose-concanavalin A while peak C contains a fraction which is not retained by Sepharose-concanavalin A and that the unretained portion of transferrin on Sepharose-concanavalin A is composed of two distinct

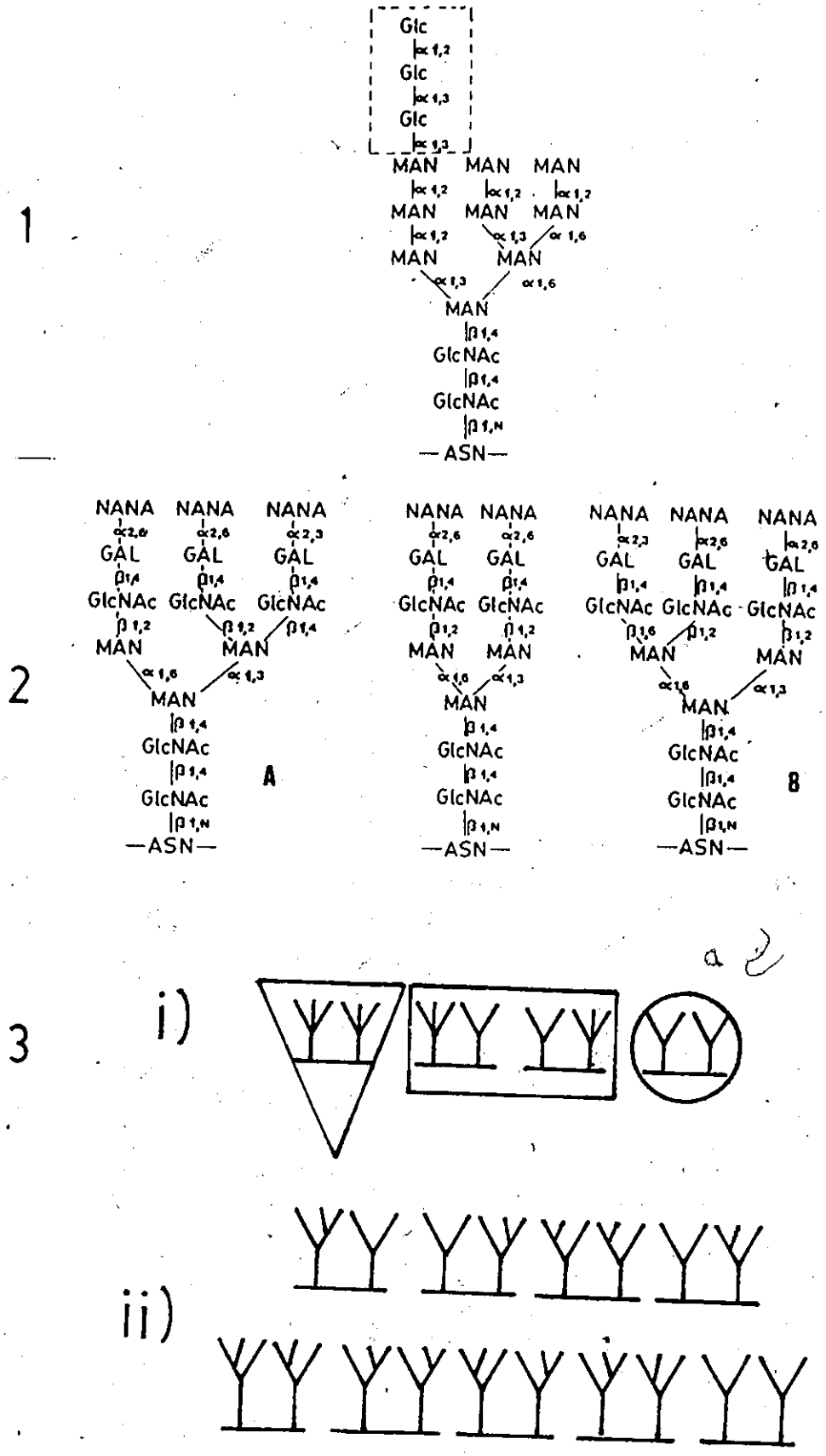

1
Glc
Glc
Glc
MAN
MAN
MAN
GlcNAc
GlcNAc
—ASN—
2
NANA
GAL
GlcNAc
MAN
A
B
3
i)
ii)

FIGURE 1.3

FIGURE 1.3 1. The complete oligosaccharide structure transferred to the protein at the endoplasmic reticulum. The residues pictured within the box are trimmed before the glycosylated protein is moved to the Golgi apparatus for further modifications. 2. The three forms of N-linked complex glycan found on human transferrin. A and B represent the two types of triantennary glycan while the standard biantennary glycan is depicted in between. 3. (i) The known arrangements of tri- and biantennary glycans on human transferrin. (After Hatton and Berry, 1984) See text for an explanation of symbols. (ii) All potentially possible arrangements of glycans on human transferrin (as inferred from present knowledge of glycan structures).

fractions: a first peak which is unretarded and a second peak which is retarded by Sepharose-concanavalin A (Wong et al., 1978). Hatton and Berry (1984) demonstrated that the first peak contains mostly two triantenpary glycans (see Figure 1.3, (2) - A and B, and (3i)- figure in inverted triangle). The second peak contains bi- and triantennary glycans expressed on transferrin in at least two forms: one form containing a biantennary (site 1) and a triantennary (site 2) glycan and a second form bearing triantennary (site 1) and biantennary (site 2) glycans (see Figure 1.3, (3i)- figures in squares). The Sepharose-concanavalin A retained material was 90% transferrin containing two biantennary glycans (see Figure 1.3, (3i)- circled figure, and (2)- middle glycan). This type of transferrin is the most predominant form. The remaining 10% contained triantennary (site 1) and biantennary (site 2) glycans with a minor amount of biantennary (site 1) triantennary (site 2).

The chemical composition of the glycans has been worked out by Spik et al. (1975, 1985). From this work it appears that there are two types of triantennary glycan illustrated with the letter A beside one and B beside the other in Figure 1.3 (2). (The structure in between these two is a biantennary glycan also found on human transferrin.) The relative proportionality of

these triantennary glycans is approximately 45:55 (A:B) within a pooled sample of transferrin known to contain only triantennary glycans (Spik et al., 1985). However, it is not known whether there are two triantennary glycans of the same type on transferrin or if type A and type B are on the same molecule (and if type A and B are situated on the same molecule what proportion of type A and B glycans are at site 1 and site 2). Furthermore, it isn't known whether the transferrins containing bi- and triantennary glycans have type A or type B triantennary glycans (or a mixture). Potentially all variations are possible although not proved. (See Figure 1.3, (3ii) for an illustration of all of these possibilities.)

The significance of this degree of heterogeneity of glycan expression in transferrin, not to mention in all glycoproteins, is a question which has tantilized investigators for a number of years. However on the whole, glycans have been viewed mostly as performing only general roles such as protection for survival from proteases (West, 1986) and in particular investigators have really only examined the gross effects of glycosylation as opposed to looking at the impact of micro-heterogeneity. For example, it has been shown in primary cultures of rat and chick hepatocytes that in the presence of tunicamycin (a compound which interferes with the initial transfer in the pathway of synthesis of the

precursor oligosaccharide (Elbein, 1984)) there is a subtle decrease in the synthesis of transferrin but in spite of the absence of a glycan, the molecule is secreted at much the same rate as glycosylated transferrin (Struck et al., 1978). This has also been demonstrated in pregnant rabbit mammary gland explants where a lack of prolactin results in non-glycosylated, but equally well secreted transferrin (Bradshaw et al., 1985). Finally, Kornfeld (1968) produced three different preparations of human transferrin: 1. 100% of the sialic acid removed by neuraminidase; 2. 39% of carbohydrate removed using glycosidases and; 3. 47% of carbohydrate removed from the glycan using glycosidases. Upon incubation of each of these preparations with reticulocytes <u>in vitro</u> he found: 1. no effect; 2. a doubling and; 3. a slight increase in iron donating capacity respectively, which, was concluded to not be significant. In a further experiment, the isolated glycan structure from human transferrin was used as a competitor to holotransferrin in similar uptake studies; again no effect was found.

Aside from such studies, little has been done to examine the impact of transferrin's glycan on its physiological function and next to nothing has been done in respect of the physiological consequences of its

microheterogeneity. In order to address the issues of specific role(s) for the glycan in human transferrin, a starting hypothesis is that specific differences between glycan structures should be attributable to specific physiological consequences in terms of function, for example, delivery of iron to cells. However, the complexity and considerable microheterogeneity of glycans on human transferrin (as outlined above) provides a formidable if not impossible obstacle for its use as a model to easily attribute function to structure. Rat transferrin on the other hand has been demonstrated to contain only one glycan and to express what has been suggested as a straightforward pattern of glycosylation; only two subforms based on glycosylation were thought to exist (Schrieber et al., 1979). Given this model, it seemed feasible to attempt to determine what impact the variation in a single glycan might have on the physiology of transferrin. With this objective in mind, studies on the resolution of rat transferrin into its constituent subforms and the effect they have on transferrin's functions formed the focus of work for the next five chapters of the present volume.

1.4 Transferrin as a Growth Factor

As indicated above, transferrin synthesis is stimulated in the testis by factors such as EGF and testosterone both of which are growth factors. The supposition that transferrin itself may be a growth factor is a recent idea. This notion has itself spawned many studies which suggest that without transferrin, those cells studied (see note 1.20 for the particular cell types) do not replicate, divide, or survive. In some cases it is the presence of the transferrin molecule itself which appears to be important while in others it is simply the availability of iron. It may well be that specific subcellular routing of iron is important and the route is sensitive to transferrin as opposed to iron alone. On the other hand, it could simply be that the iron needs and methods of presentation of that iron may differ between the many cell types studied, and which occur in the living organism.

1.5 Iron Metabolism

Iron in aqueous solution has two stable oxidative states: Fe^{2+} and Fe^{3+} and because of this it participates in many bioenergetic pathways where flux of electrons is necessary. Most of the total body's iron (2/3 to 4/5) is found in red blood cells (Huebers and Finch, 1987) complexed to hemoglobin, iron not used by these cells is

stored in ferritin (Grasso et al., 1984). The total body content of iron in the adult human amounts to between 2 to 5 grams (depending on sex and body size). Iron is carried in the plasma by transferrin which is equally distributed between the intra- and extravascular fluids (Huebers and Finch, 1987). The number of iron atoms which are delivered to the tissues is found to increase with increasing plasma iron and transferrin saturation, however the number of iron bearing transferrin molecules that leave the plasma per unit time to bind to tissue receptors is relatively constant and is independent of transferrin saturation (Cazzola et al., 1985). Iron loss occurs mostly in the gastrointestinal tract in the forms of fecal blood loss, biliary excretion, shedding of epithelial cells and insignificant losses occur in sweat and urine (Refsum and Schreiner, 1984). A scheme of plasma iron turnover is presented in Figure 1.4. In normal, non-anaemic individuals absorption of iron across the intestinal epithelium[1-21] is closely related to the total amount of storage iron regardless of the plasma iron concentration. In the case of constant plasma turnover any decrease in storage iron must be balanced by an increased transfer from the intestine, i.e., increasing absorption in order to maintain equilibrium (see Figure 1.5) (Jacobs, 1980).

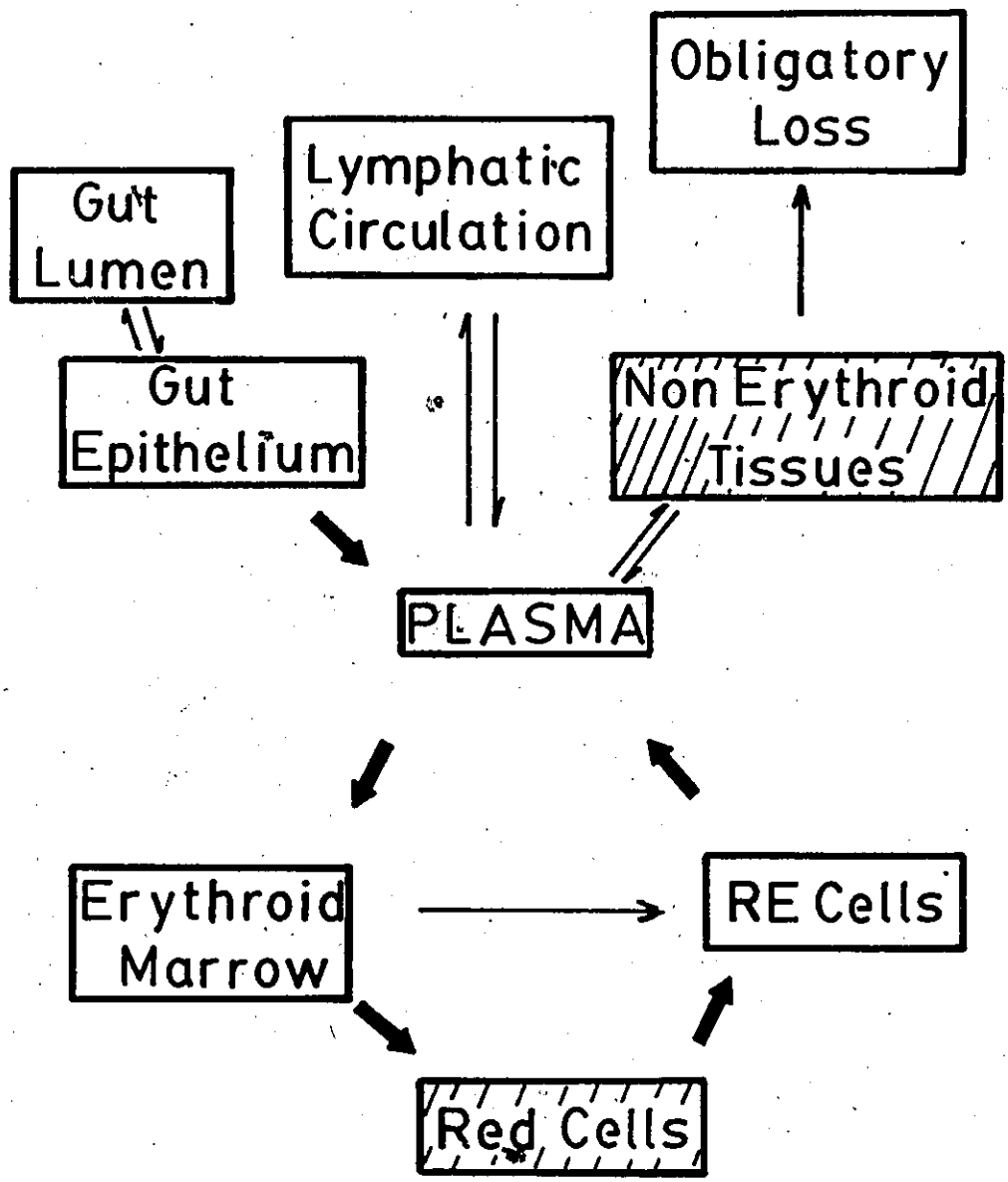

Obligatory Loss
Lymphatic Circulation
Gut Lumen
Gut Epithelium
Non Erythroid Tissues
PLASMA
Erythroid Marrow
RE Cells
Red Cells

FIGURE 1.4

FIGURE 1.4 A biologic model for pathways of iron turnover. The arrows leading to and from the plasma compartment depict pathways of iron turnover, the heavier arrows are representative of the bulk of the iron. Iron which remains localized in tissue is denoted by the shaded areas. Non-erythroid tissues includes the parenchymal cells of the liver. The total reflux consists of a relatively short sojurn in the lymphatic circulation and a longer stay via the reticuloendothelial cell (RE Cells) from non-viable erythrocyte precursors. (After Cook et al., 1970 and Jacobs, 1980)

Up to 15 mg/kg of body weight is represented as surplus iron (Huebers and Finch, 1987). The site to which this iron is sequestered is an apparently universal, ubiquitous (Crichton, 1984) iron storage protein called ferritin.[1-22] It appears that in many tissues, iron which crosses the cell membrane is initially transferred to an intermediate cytosolic pool. This pool is in a dynamic equilibrium with replenishing extracellular sources as well as the depleting sites such as: synthesis of iron proteins, incorporation into ferritin and, return to the extracellular space by transferrin (Jacobs, 1980). The mechanism(s) of iron entry into a cell just alluded to, has been the subject of much investigation, the highlights of which are presented below.

1.6 Mechanisms of Diferric Transferrin Uptake

Most iron present in the plasma of a healthy individual exists in the ferric form chelated by the protein transferrin. Traces of lactoferrin and hemoglobin are known to exist in the plasma and in some clinical disorders such as thalassemia, non-transferrin plasma iron can be substantial (Huebers and Finch, 1987) however the bulk is normally in the form of diferric or monoferric transferrin. All living cells appear to require iron for survival (Newman et al., 1982) and many must acquire iron from transferrin.

Cells are able to acquire materials from the external environment by a number of means which include simple diffusion, transporter systems and a general process called endocytosis. It has been suggested that there are a number of categories to describe endocytosis (Besterman and Low, 1983) and two broad classifications are phagocytosis and pinocytosis. The former term usually describes the "cell eating" of large particles by cells and has been studied best in macrophages (Steinman et al., 1983; Cohn and Steinman, 1982). Pinocytosis, on the other hand, refers to uptake of smaller materials such as plasma proteins and other solutes. This category is divided into: a) fluid-phase pinocytosis, and includes materials which enter the cell only in the fluid content of the volume uptaken, and b) adsorptive endocytosis which involves the material which is taken up by the cell bound in some way to the surface. This latter category has been further divided into non-specific and specific adsorption (Besterman and Low, 1983; Hopkins et al., 1985). Specific adsorption is so named for the fact that many compounds in the living system have evolved complementary receptors on cell surfaces which allow for concentrated uptake of a particular material.

Most cells have evolved a receptor which is capable of binding specifically with transferrin and it

is this interaction which is widely believed to precede accumulation of iron from transferrin. The cell most commonly used to study the mechanism of receptor-mediated endocytosis (RME) of transferrin has been the reticulocyte (or a member of its lineage, for example, K562 cells; Klausner et al., 1983). However, there has been evidence accumulating to suggest that uptake of iron by hepatocytes may proceed by alternative routes such as fluid-phase and non-specific adsorptive pinocytosis, in addition to RME (Cole and Glass, 1983; Page et al., 1984; Thorstensen and Romslo, 1984a; and Trinder et al., 1986).

A major thrust of the present thesis work entails examination of the interaction between transferrin and the liver, in vivo, and hepatocytes, in vitro. Therefore, it is important that the reader be familiar with each of the uptake processes just outlined and which have been suggested to be important in transferrin uptake by the liver. Each of these will now in turn be examined in further detail.

1.6.1 Receptor-Mediated Endocytosis

It is generally believed that RME is the major route of entry to cells by most ligands (Steinman et al., 1983). It involves the clustering of a number of

receptors, which were initially distributed over the cell surface, into specialized areas known as coated pits (Helenius et al., 1983). The coated pit obtains its name from the fact that it represents a slight invagination in the cell surface and its cytoplasmic side is "coated" with the protein, clathrin (May and Cuatrecasas, 1985). The pit forms an invagination and is pinched off forming what has been variously called a receptosome (Pastan and Willingham, 1983) or pinocytotic coated vesicle (Helenius et al., 1983; Petersen and Van Deurs, 1983). How the receptors are clustered to the pit is not known. In the case of some receptors it is believed to be due to the presence of ligand (Beguinot et al., 1984; Pearse and Bretscher, 1981; and McArdle and Morgan, 1984) while in other cases, clustering appears to occur in the absence of ligand (Brown et al., 1983). Regardless of how the receptors get into the pits, subsequently the coated endocytic vesicles appear to rapidly lose their clathrin coats and fuse with endosomes of the cellular interior (Helenius et al., 1983). It is at this stage or shortly before that uncoupling of the receptor and ligand occurs in a region which has been called the compartment of uncoupling of receptor and ligand or CURL (Dautry-Varsat, 1986). Beyond this stage endosomes either return to the cell surface, move to the Golgi, or ultimately fuse with

lysosomes (see Figure 1.5) (Steinman et al., 1983). Most ligands are delivered to the lysosome which results in destruction of the protein, however the receptors themselves escape this fate and are recycled to the cell surface for further use. The transferrin- transferrin receptor system is somewhat unique in this respect in that both the receptor and the ligand are internalized and then returned to the cell surface together (Klausner et al., 1983; Dautry-Varsat, 1986).

Because of the prominence of the role played by the receptor in mediating transferrin uptake and its unique ability to return the ligand to the cell surface, a detailed examination of the receptor and its proposed mechanism of transferrin delivery follows.

1.6.1.1 The Transferrin Receptor

1.6.1.1.1 Synthesis and Regulation

The transferrin receptor has been isolated from a number of sources (see Chapter 7). It is composed of two homologous glycoproteins each containing 760 amino acid residues (McClelland et al., 1984; Schneider et al., 1984) and with a molecular weight equivalent to 90 000 Da (Stearne et al., 1985; Newman et al., 1983) (see Figure 1.6). The monomers are bonded by disulphide bridges

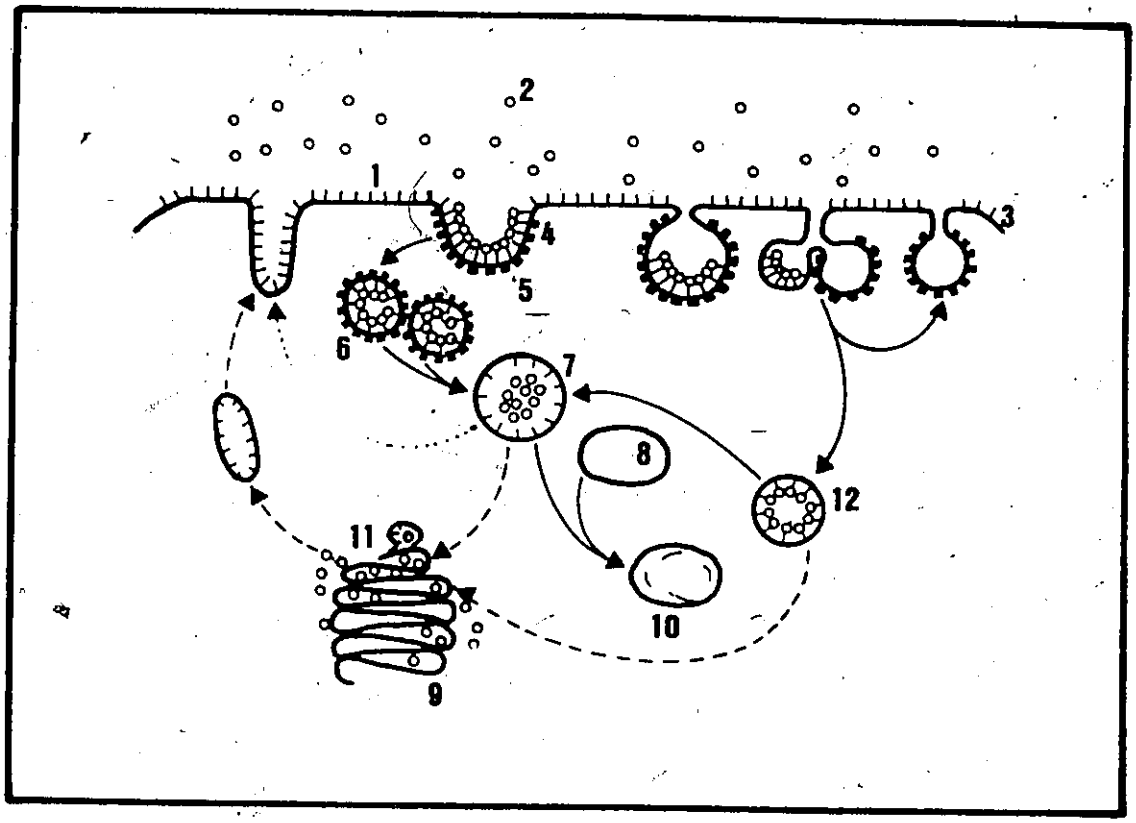


FIGURE 1.5

FIGURE 1.5 Models for receptor mediated endocytosis. 1. Receptors; 2. Ligands; 3. Plasma Membrane; 4. Clathrin; 5. Coated Pit; 6. Coated Vesicles; 7. Endosome; 8. Lysosome; 9. Golgi Apparatus; 10. Degradation of Ligands and/or Receptors; 11. Compartment of Uncoupling of Receptor and Ligand and; 12. Receptosome. Various pathways are: ——▶ Internalization of receptor and ligand via either receptosome or endosome - and fusion with a lysosome. — — —▶ Processing of either the receptosome or endosome contents at Golgi with recycling of receptors to the cell surface. ·····▶ Internalization of receptor and ligand in an endosome and recycling of receptor (and ligand in the case of transferrin) back to the cell surface. (After Morley and Bezkorovainy, 1985)

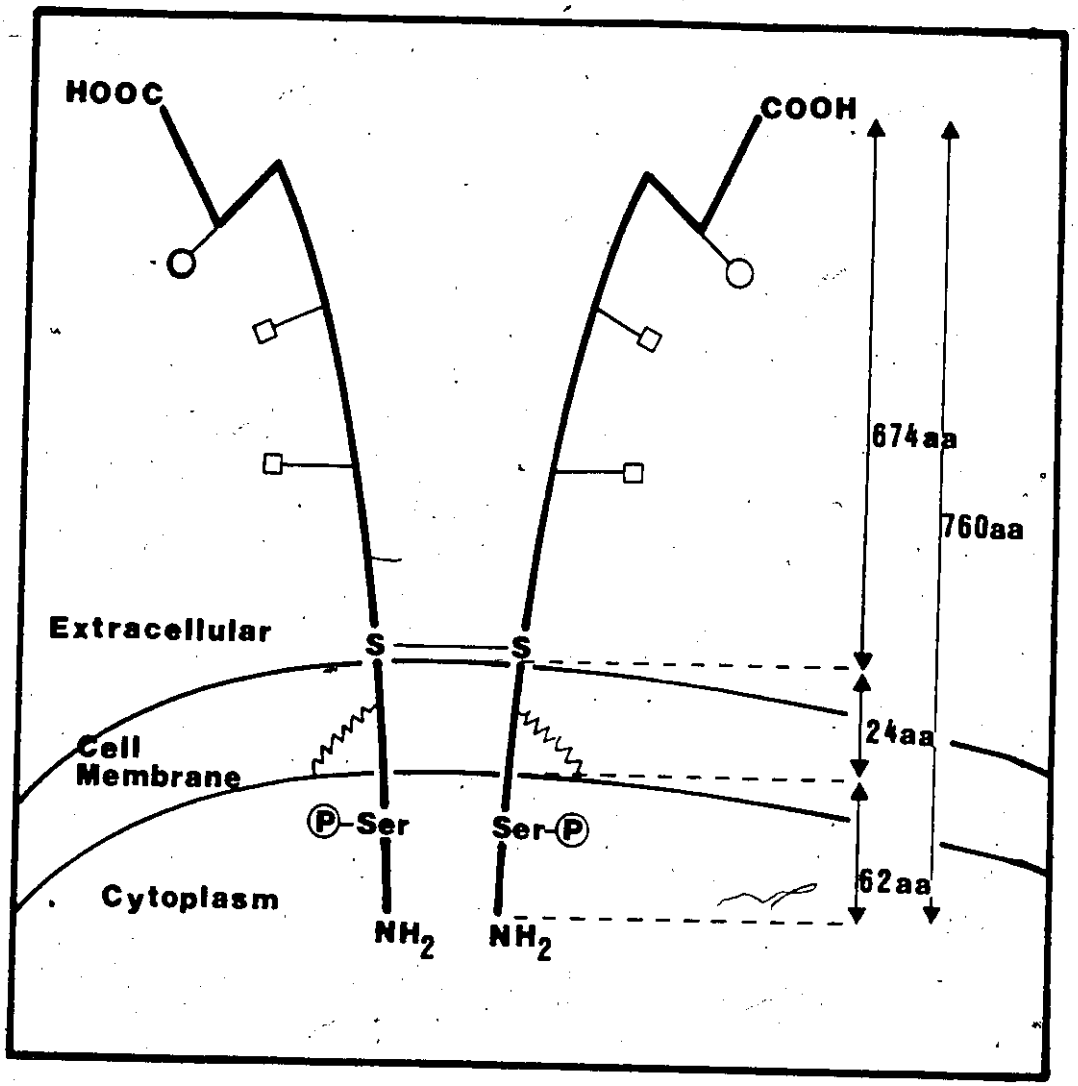

HOOC
COOH
674aa
760aa
Extracellular
S
S
24aa
Cell
Membrane
Ⓟ-Ser
Ser-Ⓟ
62aa
Cytoplasm
NH2
NH2

FIGURE 1.6

FIGURE 1.6 Schematic representation of the transferrin receptor. Each arm corresponds to a 90 KDa monomer composed of 760 amino acids (aa). The extracellular portion is 674 aa in length. Contained in the membrane is a segment 24 aa in length and 62 aa protrude into the cytoplasm. The disulphide bonds are represented by S-S and the Ser-Ⓟ corresponds to the sites of phosphorylation; □, high mannose oligosaccharide chains; ◯, complex oligosaccharide chain and ~~~ acylated fatty acid moiety. (After May, Jr. and Cuatrecasas,1985)

between corresponding amino acids at sites 89 and 98 (Jing and Trowbridge, 1987). A single hydrophobic span of amino acids, beginning at amino acid number 61 (McClelland et al., 1984) or 62 (Schneider et al., 1984) up to amino acid 89, appears to be the intramembrane region.[1-23] The remaining 672 amino acids which contain the C-terminus extends into the extracellular medium (Schneider et al., 1984). Therefore only a small portion of the receptor protrudes into the cytoplasm (5 000 Da) (Newman et al., 1983).

A site of phosphorylation has been identified at serine 24 of the cytoplasmic domain (Davis et al., 1986) and a covalently bonded fatty acid is present at cysteine 62 (Jing and Trowbridge, 1987). The purpose of this acylation, which occurs post-translationally, is not clear (Adam et al., 1984). However, the phosphorylation by protein kinase C may be related to internalization (Iacopetta et al., 1986) and/or receptor "shedding" (Adam et al., 1986) from maturing reticulocytes (Adam and Johnstone, 1987).

From studies with Endo-H it is thought that the monomers each contain three sites of glycosylation; two with high mannose glycans and the third with a complex glycan (Newman et al., 1983). Glycosylation does not appear to be important in the binding

of transferrin; however, the efficiency of cell surface expression of the receptor in tunicamycin treated cells is significantly reduced (Ward, 1987).

It is clear that growing cells have higher metabolic requirements than their adult counterparts and therefore have a greater need for iron.[1-24] As indicated above, it has been shown that phosphorylation of the receptor by protein kinase C appears to be involved in its expression on the cell surface[1-25] and control over the activity of protein kinase C may somehow be integrated with the cell's iron and heme content.[1-26] Perhaps it is not surprising that a transferrin receptor whose role it is to attract iron-carrying transferrin is ultimately regulated by the iron which the cell needs.

1.6.1.1.2 The Mechanism of Iron Delivery

It is widely believed that the first step in iron delivery by transferrin to a cell is the binding of ligand to receptor. Diferric transferrin has a higher affinity for the receptor than either monoferric form or apo transferrin at pH 7.4 (Huebers et al., 1985; Baker et al., 1982); the association constant (K_a) is in the order of 2×10^7 to 2×10^8 litres/mole (Young and Bomford, 1984). Subsequent to this binding, different, although not mutually exclusive theories have been proposed to

account for the release and internalization of the iron.

I. DIACYTOSIS

This term, first coined by Tolleshaug et al. (1981), described the rapid recycling of asialo-transferrin and presumably a vesicle formed from the surface membrane, to internalization, and subsequent rejoining the cell surface, and exocytosis of vesicular contents (see Figure 1.7). Diacytosis, coupled with the very unique properties of transferrin binding to the receptor and affinity for iron at various pH values appeared to be the complete explanation of iron delivery. The mechanism in detail reveals that diferric transferrin has high affinity for the receptor at pH 7.4, the predominant pH value in the plasma. As endocytosis begins, and endosome formation occurs, the pH within the vesicle is known to fall to a value of 5.0 - 6.5 (Geisow and Evans, 1984). It is also known that at a pH of less than 5.0, two atoms of iron are released from transferrin in vitro (Aisen and Listowski, 1980). The iron is suggested to be released in the environment of this acidic non-lysosomal compartment (Lamb et al., 1983). It had been thought that transferrin had to travel through the acidic lysosomes in order to release its iron (Octave et al., 1981) however this appears not to be the case. Transferrin, now in its apo state does not, like most

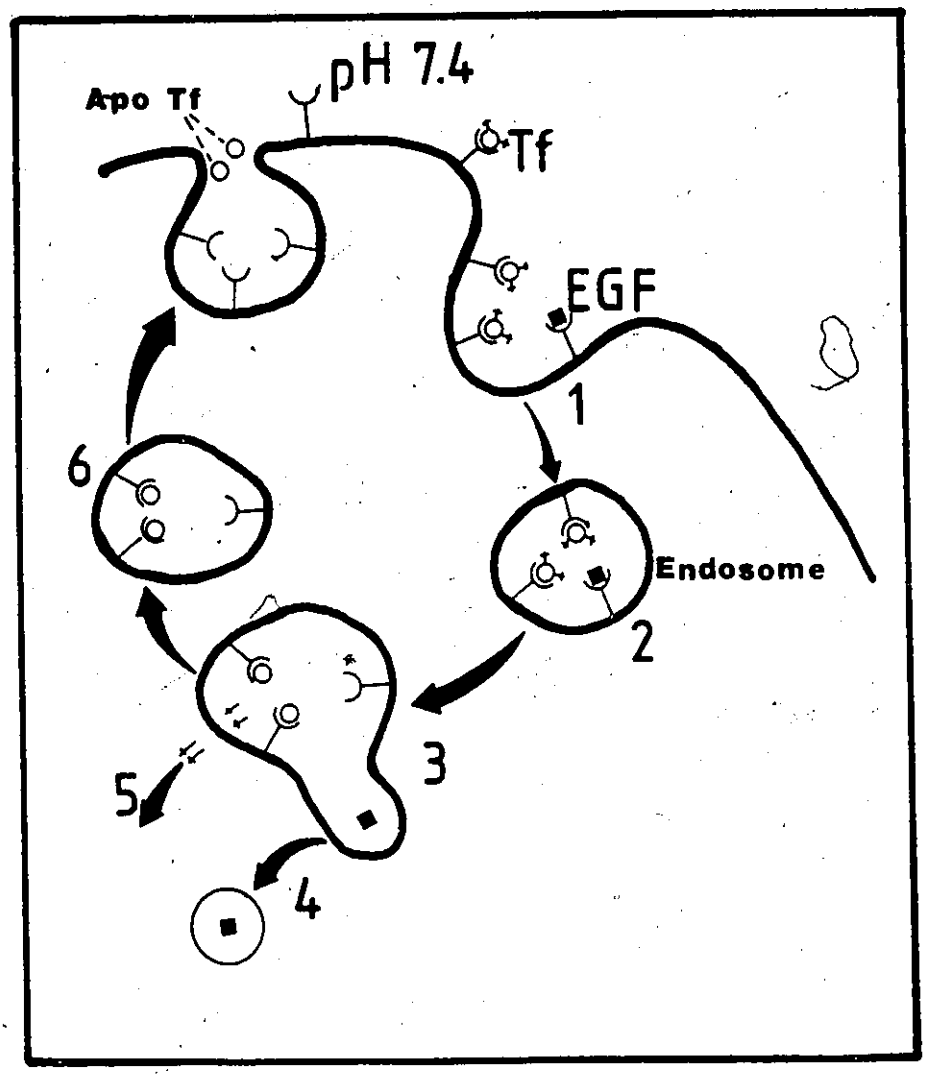


FIGURE 1.7

FIGURE 1.7 Endocytic cycle of transferrin (Tf) and of a typical ligand destined for the lysosomes (epidermal growth factor - EGF). 1. Both ligands bind to specific receptors which migrate to clathrin-coated pits. 2. Endocytosis follows with formation of an endosome and acidification of the endosomal interior by a proton pump to ~pH 5.0. 3. As a consequence of the reduced pH, iron is released from Tf and EGF dissociates from its receptor. 4. EGF is directed to the lysosomes for proteolysis. 5. The released iron becomes incorporated into ferritin. 6. The EGF receptor and apo transferrin (Apo Tf) still bound to its receptor cycle back to the plasma membrane. In the alkaline environment at the cell surface apo Tf dissociates from the receptor. (After Bomford and Munro, 1985)

other ligands do (Steinman et al., 1983; Bomford and Munro, 1985), dissociate from its receptor at this low pH and ultimately become degraded. Rather, it remains firmly bound to the receptor and recycles with the receptor back to the surface. Upon meeting the mildly alkaline environment of the extracellular fluid, due to apo transferrin's lower affinity for the receptor at this pH, the transferrin is released to the circulation (possibly in order to acquire more iron)(Dautry-Varsat et al., 1983; Klausner et al., 1983; and Slordahl et al., 1984). The receptor is once again ready to accept another diferic transferrin molecule.

Evidence to support the theory of a pH-decrease-mediated iron release from transferrin has been provided by studies of the effects of chemical modification of histidine in transferrin (Thompson et al., 1986) and by studies which measure the decrease in iron uptake in the presence of lysosomotropic drugs such as chloroquine (Octave et al., 1981). Support has also been derived from an elegant study involving examination of iron uptake by a chinese hamster ovary cell mutant which was unable to acidify endosomes. The binding and internalization of transferrin proceeded normally however, iron was not left behind (Klausner et al., 1984). Recently however, doubt has been raised as to the effectiveness of a decrease

in pH alone, to release iron from transferrin. Bakkeren et al. (1987) have shown that transferrin did not release its iron in endosomes, isolated from K562 cells which were still capable of internal acidification. However the iron was released upon incubation with the iron chelator pyridoxal or isonicotinoyl hydrazone. Thus, discussion of the second major proposal to explain iron release from transferrin follows.

II. CELL SURFACE RELEASE OF IRON

A view consistent with all theories which explain uptake of iron from transferrin is that of initial binding of diferric transferrin to the transferrin receptor. However, studies from Garrett et al. (1973), Van der Heul et al. (1978) and more recently by Glass et al. (1980) have demonstrated the presence of a macromolecule or binding protein in the plasma membrane of reticulocytes, but not red blood cells (Garrett et al., 1973), which binds the iron released from the transferrin protein. Nunez and Glass have demonstrated the presence of released iron in the plasma membranes, the cytosol and mitochondria (1983).

While these findings are not in conflict with the pH-mediated-iron release theory, work by Glass and Nunez (1986) with the amines, n-butylamine and NH_4Cl, is in

conflict. These two amines had previously been demonstrated to be capable of inhibiting iron uptake by reticulocytes, and their effect had been explained on the basis of their ability to inhibit the acidification of intracellular vesicles. However, Glass and Nunez were able to show that the actual effect was on the rate of transferrin internalization, the rate of externalization of internalized transferrin and on the transport of iron (which was released equally as well as in the absence of the compounds) across the cell membrane. Further, Smit et al. (1984) who worked with Chinese hamster fibroblasts, have calculated the there is not sufficient time for transferrin to enter cells in order to account for their observed rates of iron uptake.

The alternative explanation to account for release of iron is a reductase system (Thorstensen and Romslo, 1984b; Morley et al., 1983; Low et al., 1986 and 1987; and Nunez et al., 1983). The mechanism envisaged is either: 1) the iron binding protein has a higher affinity for the ferric iron of transferrin and releases the ferric iron which it carries across the cell membrane. At the cytosolic side the ferric iron is reduced and released to the cytosol or 2) the iron binding protein reduces the iron on transferrin and the released ferrous iron is transported across the cell membrane and is

released to the cytosol in the ferrous form (Morley and Bezkorovainy, 1985; Nunez et al., 1983).

Although this mechanism does not absolutely require endocytosis of the transferrin-transferrin receptor complex (which would accomodate the findings of Smit et al. (1984) mentioned above), there appears to be no compelling reason why the complex couldn't be internalized, indeed, there is considerable evidence supporting the proposition that it is internalized, at least in K562 cells (Enns et al., 1983), HeLa cells (Iacopetta et al., 1983) and liver cells (Sibille et al., 1986; Morgan et al., 1986).

1.6.1.1.3 Subcellular Routing of the Receptor

As much as 30% of the cell's surface transferrin receptors may be located in coated pits at any one time (Cheng, 1986) and while occupation of the receptor by transferrin may be important for clustering and internalization (Enns et al., 1983), it is not, on the balance of the evidence (Watts, 1985; Hanover et al., 1985; and Ajioka and Kaplan, 1986) a necessary condition.

Once internalized, the receptor-containing vesicle may fuse with a multivesicular body (Iacopetta et al., 1983), be quickly recirculated to the cell surface, (Stein and Sussman, 1986; Wedman et al., 1987)

or cycled to the Golgi and ultimately back to the cell surface (Stein and Sussman, 1986; Woods et al., 1986; and Fishman and Fine, 1987), or move to the lysosomes for receptor and/or ligand degradation (Helenius et al., 1983).

The subcellular route via the Golgi, which has been suggested to represent a minor pathway (Ajioka and Kaplan, 1986), is referred to as a long circuit lasting 1-2 hrs (Hedman et al., 1987). In this pathway transferrin receptor has been localized in most or all Golgi subcompartments (Woods et al., 1986) and may fulfill a role in delivering transferrin for resialylation (Fishman and Fine, 1987).

The final pathway of the transferrin receptor in reticulocytes, as they mature, appears to differ from that of normal lysosomal degradation. In these cells the transferrin receptor has been demonstrated to be "shedded" (Pan et al., 1983) in vesicles; an event which may be signalled by an inability to be phosphorylated (Johnstone et al., 1984).

While cells such as reticulocytes, HeLa cells, fibroblasts and HL-60 cells appear to depend significantly on the transferrin receptor to mediate the uptake of iron from transferrin, as suggested above, it is not clear that this is the situation for adult hepatocytes.

Indeed, at least three mechanisms have been illuminated as contributing to the uptake of transferrin (Irie and Tavassoli, 1987). The first is the transferrin receptor and it is believed to operate much as described above (Nunes et al., 1986; Baker et al., 1982 and 1985) and has been suggested to be responsible for most of the iron uptake (Thorstensen and Romslo, 1984a; Young and Bomford, 1984; Young and Aisen, 1980; and Trinder et al., 1986). The second mechanism involves non-specific adsorptive endocytosis mediated by low affinity sites (Cole and Glass, 1983; Trinder et al., 1986; and Young et al., 1983) which can be blocked by albumin (Thorstensen and Romslo, 1984b) but have been demonstrated in binding assays as revealed by Scatchard plot analysis (Trinder et al., 1986; Young et al., 1983) and have been suggested to account for as much as 50% of the uptake (Trinder et al., 1986). The third, fluid-phase pinocytosis (which will be discussed in greater detail below), has been suggested as a means of uptake but is believed to account for only less than 5% (Trinder et al., 1986) to as much as 20% (Cole and Glass, 1983). However, at least one group (Sibille et al., 1982) has suggested that this process is predominantely reponsible for mediating uptake of iron from transferrin.

A minor fourth route, which must be included, recognizes that transferrin deficient in sialic acid has been demonstrated in the plasma of normal individuals and is known to be more prevalent in conditions of alcoholism (Stibler et al., 1984). The asialoglycoprotein receptor (ASGP-R) is able to bind asialotransferrin and is therefore a second receptor potentially capable of mediating uptake of transferrin and contributing to iron accumulation in the liver. (Clearly depending on the condition of the organism this may or may not be a significant route.)

The central theme of this thesis work is the examination of the interaction of transferrin with the liver. As can be inferred from the above discussion with respect to the liver, the degree of participation by the individual mechanisms of iron delivery from transferrin, is less than clearly defined. Therefore, a significant part of this thesis work has been devoted to casting further light on the extent of the contribution by these mechanisms.

Finally, for the sake of completeness it must be mentioned that one group of investigators suggests that the liver endothelium mediates the uptake of transferrin

by the liver (Soda and Tavassoli, 1983; Kishimoto and Tavassoli, 1985 and 1987; Tavassoli et al., 1986; and Irie and Tavassoli, 1987). The proposal by Tavassoli and co-workers is that liver endothelial cells, which express transferrin receptors, first bind and take up transferrin. Once inside these cells, the transferrin is desialylated and released to the space of Disse, i.e., transferrin undergoes "transcytosis". Once in the space of Disse, the exposed galactose moieties have affinity for the ASGP-R known to exist on the hepatocyte surface (Ashwell and Harford, 1982). Asialotransferrin is taken up, but then probably "switches" to the transferrin receptor once the pH drops and iron is released (as can be inferred from the work with human asialotransferrin by Tolleshaug et al., 1981). This asialotransferrin bound to the transferrin receptor then undergoes resialylation and is returned to the circulation (as has been shown to occur partially; Regoeczi et al., 1982 and 1984b). The theory of Tavassoli and co-workers, while novel, has serious problems, among others, in explaining how complete sialylation occurs when it has been demonstrated to occur only partially.

In order to aid the reader's appreciation of all mechanisms believed to be involved in transferrin-liver interactions a brief description of the ASGP-R is included below.

1.6.1.2 The Asialoglycoprotein Receptor (ASGP-R)

The liver contains a receptor which recognizes galactose (Gal) and N-acetylgalactosamine (GalNAc) (Ashwell and Harford, 1982) and is located on hepatocytes (Tolleshaug et al., 1977). Each cell contains between 1 - 5 x10^5 receptors (Schwartz, 1984) with a large portion of these located intracellularly (Weigel and Oka, 1983).

The ASGP-R is an integral membrane protein and in the rat, it appears to be composed of three polypeptides of molecular weights 40-54 KDa for the major component and 55 and 62 KDa for the minor components (Schwartz et al., 1981; Harford et al., 1982) while the human receptor is only a single polypeptide of molecular weight 41 or 46 KDa (Baenziger and Maynard, 1980; Schwartz and Rup, 1983); all constituents and molecular weights as judged by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE). The receptor is a glycoprotein containing a complex glycan which, if desialylated, is capable of binding to the receptor itself and effectively blocking itself (Stockert et al., 1977).

Once a ligand is bound to the receptor on the cell surface (a process requiring calcium; Pricer and Ashwell, 1971) the complex is internalized by coated pits, which subsequently lose their clathrin coats, fuse

with endosomes and in the environment of reduced pH the ligand dissociates and is directed to the lysosomes for destruction while the receptor is recycled to the surface (Schwartz, 1984). It seems clear that the only ligand which escapes this fate (to a certain extent) is human asialotransferrin which is taken into the cell by the ASGP-R but is returned to the cell surface, probably on the transferrin receptor (depending on receptor availability in the endosome) (Regoeczi et al., 1982) due to apo transferrin's high affinity for the transferrin receptor at low pH values.

The final mechanisms which appear to be important in contributing to transferrin uptake by the liver are represented by non-specific adsorptive endocytosis and fluid-phase pinocytosis.

1.6.2 Non-Receptor-Mediated Endocytosis

I. Non-Specific Adsorptive Endocytosis

As already mentioned, non-specific endocytosis of transferrin has been suggested to account for as much as 50% of transferrin uptake in hepatocytes. Clearly, adsorption to the cell surface enhances the efficiency of uptake of a macromolecule as compared with fluid phase (Besterman and Low, 1983), yet beyond the demonstration

of low affinity binding sites (Cole and Glass, 1983; Trinder et al., 1986; Thorstensen and Romslo, 1984b; and Young et al., 1983) little has been provided in the way of experimental evidence to explain how transferrin cycles this way or is processed through the cell. The proposal of surface-associated release of iron by a reductase system could account for uptake of iron from non- specifically bound transferrin however, whether these molecules are spared from degradation by a similar, albeit less efficient means (due to lower affinity) as the high affinity transferrin receptor is at present, mere speculation.

II. Fluid-Phase Pinocytosis

Studies of pinocytosis reveal that this process is highly dynamic. Measurements in macrophages and lymphocytes suggest that their entire cell surface area is internalized once every 33 min and 120 min respectively (Steinman et al., 1983) and there is evidence which suggests that fluid-phase pinocytosis may also be initiated at coated regions of the cell (Marsh and Helenius, 1980).

Measurement of the rate of uptake by fluid-phase pinocytosis is done by use of markers which have as little affinity for the cell surface as possible. Mostly polyvinylpyrrolidone (PVP), sucrose or inulin have been

used (Besterman and Low, 1983). Another requirement of these markers is that they be taken up, retained and not degraded in the lysosomes; traditionally, substances taken up by fluid phase pinocytosis were believed to be destined to move only to the lysosomes (Lloyd and Williams, 1984). However, it is likely that none of the markers mentioned above, or any other marker for that matter, is able to completely meet these criteria. The reason for this view is the recent realization that pinosomes (initial vesicles formed just after invagination and pinching off at the cell surface) can avoid the lysosomes entirely and be quickly returned to the cell surface and release their contents (à la diacytosis) (Scharschmidt et al., 1986; Lloyd and Williams, 1984; and Besterman et al., 1981).

In order to determine the fate of the fluid taken up in a pinosome, Besterman et al. (1981) followed the path of [^{14}C]sucrose. They found at least two significant routes. The first was via a smaller compartment which rapidy turned over with a half-life of 5-8 min while the other turned over more slowly (180-620 min) (for each cycle the faster times were obtained from measurements in macrophages and the slower times from fibroblasts). Besterman et al. (1981) found that on the basis of compartment sizes and rates of

intercompartmental exchange between the pinocytotic vesicles, most of the fluid taken in was rapidly exocytosed. Similar findings have been reported in hepatocytes (Scharschmidt et al., 1986). However, many reports suggest that exocytosis of only a small amount of the pinocytosis markers such as PVP, sucrose and colloidal gold occurs with most of the material being retained by the lysosomes (Steinman et al., 1983; Lloyd and Williams, 1984) and at present the difference in views remains unresolved.

If pinosomal contents are directed to the lysosomes, then the finding in liver of a small percentage of transferrin uptake by this mechanism is probably correct (Trinder et al., 1986). Otherwise, if uptake of transferrin by pinosomes was greater, then total transferrin degradation would likely be much higher than it is known to be (see Chapter 3). In fact, given the small percentage of uptake estimated to occur by pinocytosis (<5%), this may represent the most significant pathway of transferrin turnover.

Yet, the findings of Besterman et al. (1981) and Scharschmidt et al. (1986) coupled with those of Sibille et al. (1982) suggest that in the liver, the pathway of pinocytosis may be more important in delivery of transferrin and aquisition of iron and <u>not</u> in degradation of the protein. This divergence of views underlines

the point made above about the uncertainty concerning the relative contribution of each mechanism to transferrin uptake; a major objective of this study is to cast further light on this issue.

1.7 Objectives

In the light of the overview presented above it is clear that many areas of the present understanding of transferrin are lacking. The central objective of this study was to add to the knowledge base concerning transferrin's interaction with the liver. In particular, three major objectives of the present study are encompassed in the examination of the following issues:

1. Rat transferrin exists as a number of subforms with respect to variations in its glycan structure. Are there any measureable functional, biological consequences of the glycan microheterogeneity of rat transferrin?

2. Transferrin's primary function is transport of iron, however the loading and release of this metal has been little investigated with reference to variations

in glycan structure. Does glycan microheterogeneity measurably affect the reactions of iron with rat transferrin?

3. Most cells contain transferrin receptors yet the liver, a site of iron storage, may not mediate transferrin uptake exclusively by specific receptors. How significant is the transferrin receptor in mediating transferrin-liver interactions?

The first issue was investigated and reported on in Chapters 2, 3, 5 and 6 while the second is addressed in Chapters 3 and 4. The answer to the third issue is in Chapter 9 but the search for the answer forms a thread of continuity through Chapters 7, 8, and 9.

The work presented in this thesis has formed, in part, the basis of several publications and the following is a list of these publications:

1. Regoeczi, E., P.A. Chindemi, J.R. Rudolph, G. Spik and J. Montreuil. (1987) The chromatographic heterogeneity of rat transferrin on immobilized concanavalin A and lentil lectin. Biochem. Cell Biol. 65: 948-954.
   CHAPTER 2 (The bulk of the material in this chapter was drawn from the paper.)

2. Rudolph, J.R., E. Regoeczi, P.A. Chindemi, and M.T. Debanne. (1986) Preferential hepatic uptake of iron from rat asialotransferrin: Possible engagement of two receptors. Am. J. Physiol. 251: G398-G404.
   CHAPTER 4

3. Rudolph, J.R. and E. Regoeczi. (1988) Interaction of rat asialotransferrin with adult rat hepatocytes: Its relevance for iron uptake and protein degradation. J. Cell. Physiol. 135: 539-544.
   CHAPTER 6

4. Rudolph, J.R. and E. Regoeczi. (1987) Isolation of the rat transferrin receptor by affinity chromatography. J. Chromatogr. 396: 369-373.
   CHAPTER 7

5. Rudolph, J.R., E. Regoeczi, and S. Southward. (1988) Quantification of rat hepatocyte transferrin receptors with poly- and monoclonal antibodies and protein A. Histochemistry 88: 187-192.
   CHAPTER 9

This thesis has been written by John Rudolph and the publications were written in collaboration with Dr. E. Regoeczi. Similarities and occasional parallels in the text and presentation of the thesis with the

publications was unavoidable and that fact is acknowledged here. The other authors of the publications, Dr. M.T. Debanne, Mr. P. Chindemi and Ms. S. Southward made contributions in the form of their work on particular experiments. Dr. J. Montreuil, Dr. G. Spik and their co-workers characterized the chemical structures of the transferrin glycans.

CHAPTER 2

THE MICROHETEROGENEITY OF GLYCAN STRUCTURE IN RAT TRANSFERRIN

2.1 Introduction

The N-glycans of plasma and other glycoproteins are known to be subject to considerable structural variations, usually referred to as microheterogeneity (for reviews see Berger et al., 1982 and Hatton et al., 1983). A classic example is ovalbumin where (at least) ten different structures can be present at the single site of glycosylation (Narasimhan et al., 1980; Iwase et al., 1981). As indicated in Chapter 1, Appendix CH-1, note 1.19 at least three factors appear to be involved in producing the variety of N-glycans.

A major objective of the present work was to attempt to establish whether the glycan microheterogeneity present in transferrin had any ramifications concerning its physiological role. It has been found in earlier studies with human transferrin from individual donors, that the proportion of glycan variants is remarkably constant in health (Regoeczi et al., 1977 and 1979). While this is an attractive feature in terms of obtaining pure subfractions, the presence of even just the two glycans constituted a formidable hurdle in understanding what attributes of the glycans might be

significant in the presence or absence of physiological sequels. For this reason it seemed desirable to search for a simple probe of the consequences of variable glycan structure. Rat transferrin, a protein containing a single N-linked glycan and present in only two subforms (Schreiber et al., 1979) was chosen. In order to be able to attribute functional roles to structural features it was necessary to characterize rat transferrin's glycans as completely as possible. Presented below are observations regarding the unsuspected microheterogeneity of this protein as manifested during chromatography on two lectins. As well, the complete chemical structures as determined by Dr. G. Spik and her co-workers at the Laboratoire de Chimie Biologique de Universite des Sciences et Techniques de Lille I are presented.

2.2 Experimental Procedures

2.2.1 Materials

Lens culinaris (lentil) lectin, insolubilized on Sepharose 4B (2 mg lectin per ml of packed gel; cat no. L 0511), 1-O-methyl-D-glucopyranoside, methyl-D-manno-pyranoside (grade III) were obtained from Sigma. concanavalin A Sepharose (con A), CM-Sephadex C-50 and Sephadex G-50 (fine) were purchased from Pharmacia

(Uppsala, Sweden). DEAE-cellulose was from W. & R. Balston (Maidstone, U.K.) and Desferal (deferoxamine mesylate) from Ciba. $^{59}FeCl_3$, $Na^{125}I$ and $Na^{131}I$ were obtained from New England Nuclear (Boston, MA). Monospecific antiserum to rat hemopexin was a gift from Dr. W.T. Morgan (Louisiana State University, New Orleans, LA). All chemicals used were of analytical grade where available.

2.2.2 Preparation of Rat Transferrin

The starting material was pooled blood from large Sprague-Dawley rats of either sex. They were anaesthetised with ether and exsanguinated by cardiac puncture, keeping hemolysis to a minimum. Sodium citrate or acid-citrate-dextrose was used as the anticoagulant. Plasma was separated from red and white blood cells by centrifugation at 150 g at 4°C for 10 min. Transferrin was isolated from the plasma by a three-step procedure:

1. Salt fractionation was carried out at 40% saturation of $(NH_4)_2SO_4$ with stirring for 1 hour at room temperature. This was followed by centrifugation at 10,000 R.P.M. for 20 min at 0-4°C. The supernatant was then dialysed for 18 hours against continuously changing cold distilled water. The dialysate was then titrated to pH

5.9 and an ionic strength of 1250 MOHs using 0.2 M tri-sodium citrate pH 5.9.

2. The dialysate was passed through a column (2.5 x 40 cm) of CM-Sephadex which had been equilibrated with 0.01M tri-sodium citrate, pH 5.9. Once the material had passed through the column, washing with the equilibration buffer continued until the optical density, by spectrophotometry, of the effluent was less than 0.05 at 280 nm. Step elution of bound material was effected with 0.1M tri-sodium citrate pH 5.9. Transferrin was located in the collected fractions by adjusting the pH of each fraction to 7.5 with 1M $NaHCO_3$ and addition of 50 ul of 9 mg/ml $FeCl_3$:citrate (1:4). The fractions which contained transferrin turned characteristically salmon-pink in colour. These fractions were pooled and concentrated to 2-3 ml under pressure dialysis. This volume was then re-adjusted to pH 7.5 with 1M $NaHCO_3$ and a saturating amount of Fe was added in the form of $FeCl_3$:citrate (1:4) then incubated for 4 h at room temperature. Subsequent dialysis was against 4 x 500 ml of 0.01 M Tris-HCl pH 8.0. for 18 h at 4°C.

3. The dialysis residue was loaded onto a DEAE-cellulose column (1.5 x 40 cm) which had been equilibrated with 0.01M Tris-HCl, pH 8.0. Transferrin was eluted from the column by gradient-elution. The asymptotic gradient

was generated by using a 650 ml mixing chamber containing 0.015M Tris-HCl pH 8.0 and 0.175M Tris-HCl pH 8.0, as the limit solvent. Both the CM- and DEAE chromatographies were performed at room temperature.

Saturation of transferrin with iron was calculated from the absorbance ratios of 465 nm and 280 nm (Huebers et al., 1977).

Human transferrin was prepared by a similar procedure from pooled human plasma (Regoeczi et al., 1979).

2.2.3 Chromatographies on Lectin Columns

Preparative con A chromatography was performed on a 1.5 cm x 18 cm column. The equilibrating buffer was 10 mM Tris-HCl, pH 7.4, containing 1 mM each of MgCl, MnCl and $CaCl_2$, and 0.5M NaCl. Specific elution was effected with the same buffer but containing 0.1M methyl glucopyranoside.

Columns of 1 cm x 5 cm were used for working with lentil-Sepharose. The equilibrating buffer was the same as that for con A-Sepharose except that the pH was 8.0. Methyl mannopyranoside (0.5M), dissolved in the equilibrating buffer, was used to elute proteins bound by the lectin. All lectin chromatographies were performed at room temperature.

2.2.4 Iodination of Transferrin

Transferrin, and all other proteins radioiodinated and used for work described in this thesis, were iodinated with tetrachloroglycoluril as the catalyst. The protein to be labelled (usually 50-200 ug) was contained in a volume less than 150 ul in a 12 X 85 mm glass test tube. To this was added 50 ul of Na borate buffer and 10 ul of 1 ug/ml NaI (concentration with respect to iodide, I-). This was followed by 0.5 or 1.0 mCi of radioiodide then immediately mixed in a small 3 ml glass vial which had 10 ug of the tetrachloroglycoluril coated onto the walls of the vial. (Sufficient tetrachloroglycoluril was solubilized in a volume of chloroform such that 100 ul of the solution contained 10 ug of the catalyst. 100 ul aliquots were then added to each vial, the chloroform was allowed to evaporate leaving behind a film of tetrachloroglycoluril; a stock of coated glass vials was kept for as long as three months in a refrigerator. A small piece of wire was inserted as a stir bar.). The reaction was allowed to continue, with stirring, for exactly 1 min at room temperature. The reaction was stopped by removal of the solution from the vial. The remaining free iodide (radioactive and non-radioactive) was separated from the protein either by chromatography on a 1.5 ml column of the anion exchanger

Dowex (1 X 4)(in the chloride cycle) contained in a 3 ml disposable syringe or by dialysis against 0.05M NaI in phosphate buffered saline (PBS). Essentially, this method follows that described by Regoeczi (1983).

2.3 Results

2.3.1 Chromatography of Whole Rat Transferrin on DEAE-cellulose

The present isolation procedure made possible resolution of rat plasma transferrin into two major and three minor components (Fig. 2.1). The major components, which comprised 92-94% of the total transferrin as detected by radioiron tracing, were electrophoretically homogeneous and free of hemopexin according to the results of Ouchterlony plates set up against a monospecific antiserum to rat hemopexin. Peak 4 transferrin electrophoresed in polyacrylamide gel at pH 7.7 faster than peak 3 transferrin, the difference in mobilities being consistent with the presence of an additional sialic acid in peak 4 transferrin (Fig. 2.2). These peaks 3 and 4 correspond to the "slow" and "fast" transferrins described by Gordon and Louis (1963). The major components were used for lectin affinity studies described further below. As to the minor components, the

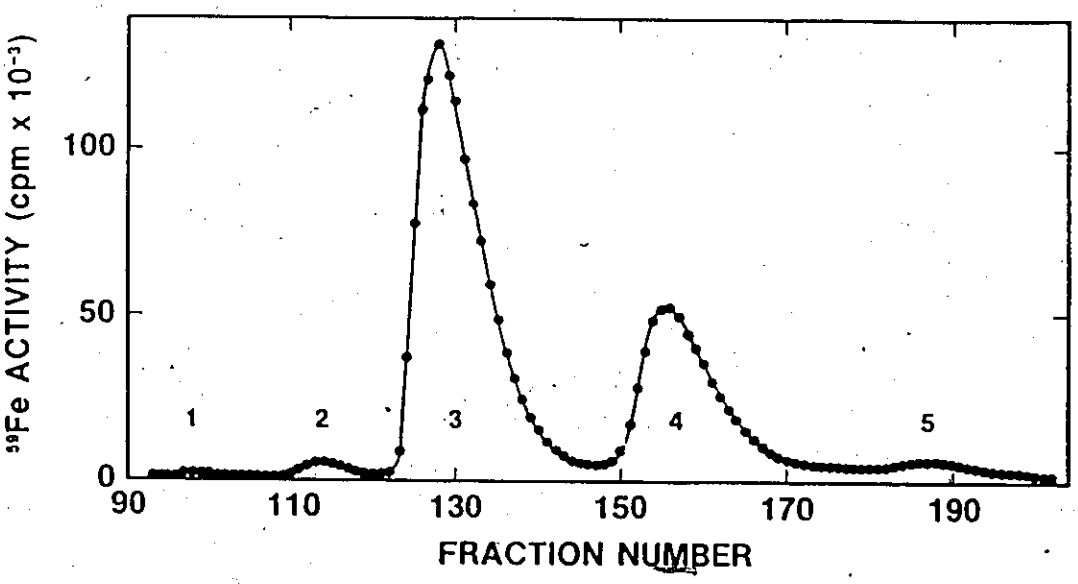

^{59}Fe ACTIVITY (cpm x 10^{-3})
100
50
0
1
2
3
4
5
90
110
130
150
170
190
FRACTION NUMBER

FIGURE 2.1

FIGURE 2.1 Chromatogram of ^{59}Fe-labelled, iron-saturated rat transferrin on DEAE-cellulose. A column of dimensions 1.4 cm x 30 cm was loaded with approx. 28 mg of transferrin. The fraction volumes were 3.6 ml. Chromatographic conditions were as described in Experimental Procedures (see section 2.2.2). The proportions (peak elution volumes in parentheses) of the five ^{59}Fe peaks seen were: 1, 0.6% (353 ml); 2, 1.7% (410 ml); 3, 63.0% (461 ml); 4, 30.8% (561 ml); and 5, 3.9% (673 ml).

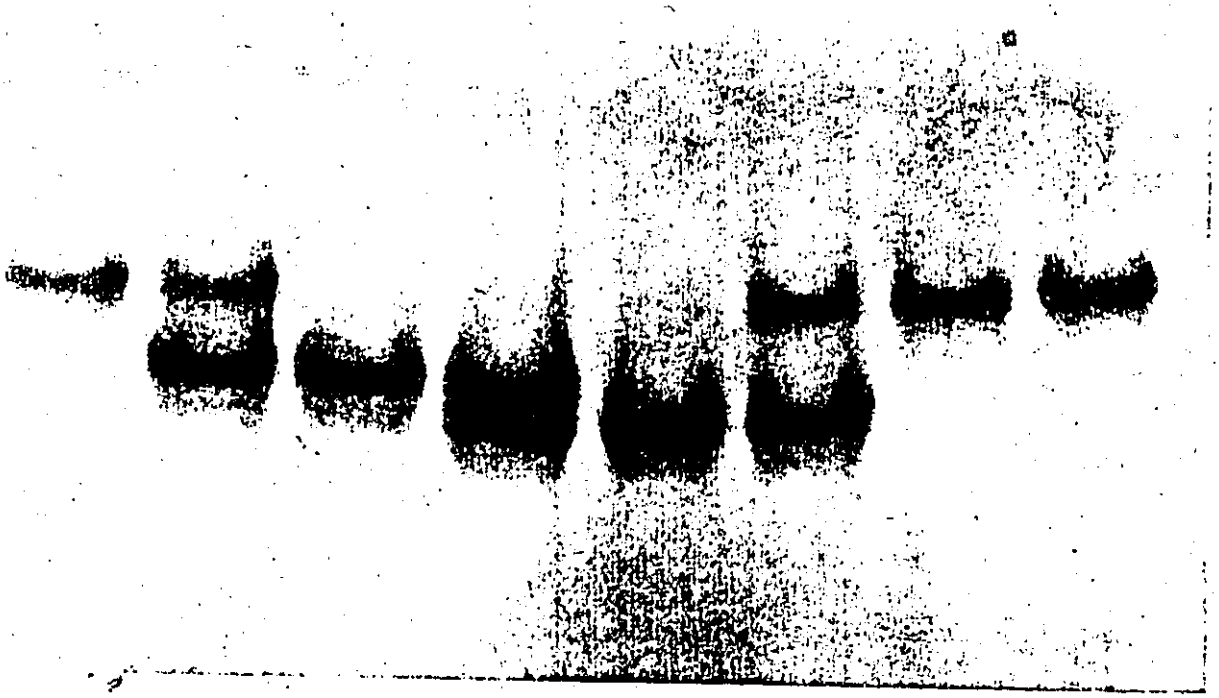


FIGURE. 2.2

FIGURE 2.2 Electrophoretograms of rat transferrin from DEAE peaks 3 and 4 in the native form before and after desialylation (see Chapter 3, section 3.2.2 for desialylation method). The alkaline (pH 7.7) polyacrylamide (7.5%) slab gel was run at constant current of 180 V for 6 h and then stained with Coomassie blue G250. Migration was from top to bottom, with the anode at the bottom. The samples, from left to right, are: (1), DEAE peak 3 desialylated transferrin; (2), mixture of DEAE peak 3 transferrin before and after desialylation; (3), DEAE peak 3 transferrin; (4), mixture of DEAE peaks 3 and 4 transferrin; (5), DEAE peak 4 transferrin; (6), mixture of DEAE peak 4 transferrin before and after desialylation; (7), DEAE peak 4 desialylated transferrin; and (8), mixture of asialotransferrins obtained from DEAE peaks 3 and 4 transferrins. Tracks were loaded with 10 to 15 ug of protein in approx. 50 ul. Note the relative spacing of the bands bearing in mind that DEAE peak 3 transferrin possesses a single biantennary glycan.

chromatographic position of peak 1 (not always detected) corresponded to that of rat asialotransferrin, and the position of peak 2 (always present) to that of monosialylated rat transferrin. However, chemical proof of these inferences could not be furnished because of the scarcity of chromatographic material. The transferrin obtained as peak 5 was contaminated with hemopexin.

2.3.2 Behaviour of DEAE-transferrin Peaks 3 and 4 on Con A-Sepharose

All studies described in this section were carried out with iron-saturated transferrin.

As was expected from the work of Schreiber et al. (1979), the transferrin available in DEAE peak 3 was fully bound by con A and it required specific elution by glucopyranoside. Any material found in DEAE peak 3 that behaved differently on con A also exhibited higher electrophoretic mobility, and was therefore regarded as contamination by peak 4 transferrin due to incomplete resolution of the DEAE peaks.

Unexpected from the literature was, however, the resolution of DEAE peak 4 transferrin on con A into a nonretarded and a retarded fraction. This resolution was observed regularly, with the relative proportions of both

fractions varying within a narrow range from one batch of protein to another (Table 2.1). The extent of retardation, expressed as the ratio of the elution volumes of the retarded and nonretarded peaks, was 1.92 (± 0.07 SD) with a range from 1.82 to 2.05.

2.3.3 Analysis of DEAE-transferrin Peaks 3 and 4 by Lentil-Sepharose Chromatography

The work with rat transferrin on the lentil-Sepharose was performed by Mr. P. Chindemi in Dr. E. Regoeczi's laboratory. This section of work was included to enable the reader to appreciate the complete picture of rat transferrin as is presently available and as an aid for thinking about the impact microheterogeneity has on biological function.

In agreement with a report by Skinner et al. (1984) which indicated that both rat testicular and serotransferrin contain fucose, Mr. Chindemi also found fucose in serum transferrin samples.

His results, in summary, suggested that each of rat transferrin's three con A-derived subfractions contained a subpopulation of molecules with affinity for lentil-Sepharose. The proportion of these molecules was close to 1 in 4 with no significant differences with respect to the form of transferrin analyzed. Re-

TABLE 2.1 Resolution of rat DEAE peak 4 transferrin of con A-Sepharose

Experiment	Load (mg)	Nonretarded (%)	Retarded (%)
1	30	83.8	16.2
2	20	84.8	15.2
3	25	83.6	16.4
4	30	85.2	14.8
5	30	82.0	17.0
6	30	85.7	14.3
7	35	89.0	11.0
8	32	81.0	19.0
9	35	82.6	17.4
10	16	83.2	16.8
11	16	85.7	14.3
12	30	85.1	14.9
13	27	84.9	15.1
14	27	84.2	15.8
15	35	83.0	17.0
16	22	88.0	12.0
17	34	84.8	15.2
Mean (± SD)	--	85.5 ± 2.0	15.5 ± 1.9

NOTE: Runs were performed on a 1.5 cm x 18 cm column. Percentages were calculated from the absorbance of the fractions at 280 nm.

chromatography of transferrin molecules retained by the lentil column (after removal of the eluting sugar by dialysis) resulted in a quantitative recapture of the load. Human transferrin (the type which possesses two biantennary glycans), in contrast to rat transferrin, showed no affinity for lentil lectin.

The three con A-derived fractions and their fucose containing counterparts were isolated in bulk by Mr. Chindemi. Sufficient quantities of each type were sent, for the determination of their glycan structures, to the Laboratoire de Chimie Biologique de Université des Sciences et Techniques de Lille I. Final representations of the structures are illustrated in Fig. 2.3.

2.4 Discussion

The heterogeneity of rat transferrin (two forms) has been known for some time (Gordon and Louis, 1963; Huebers et al., 1977; and Morgan et al., 1978). Gordon and Louis analyzed the carbohydrate composition of both forms quantitatively, but found no difference. However, Schreiber et al. (1979) found that an additional sialic acid was present in the type of transferrin not bound by con A, while the other type, which comprised the greatest proportion of serum transferrin, was bound to con A.

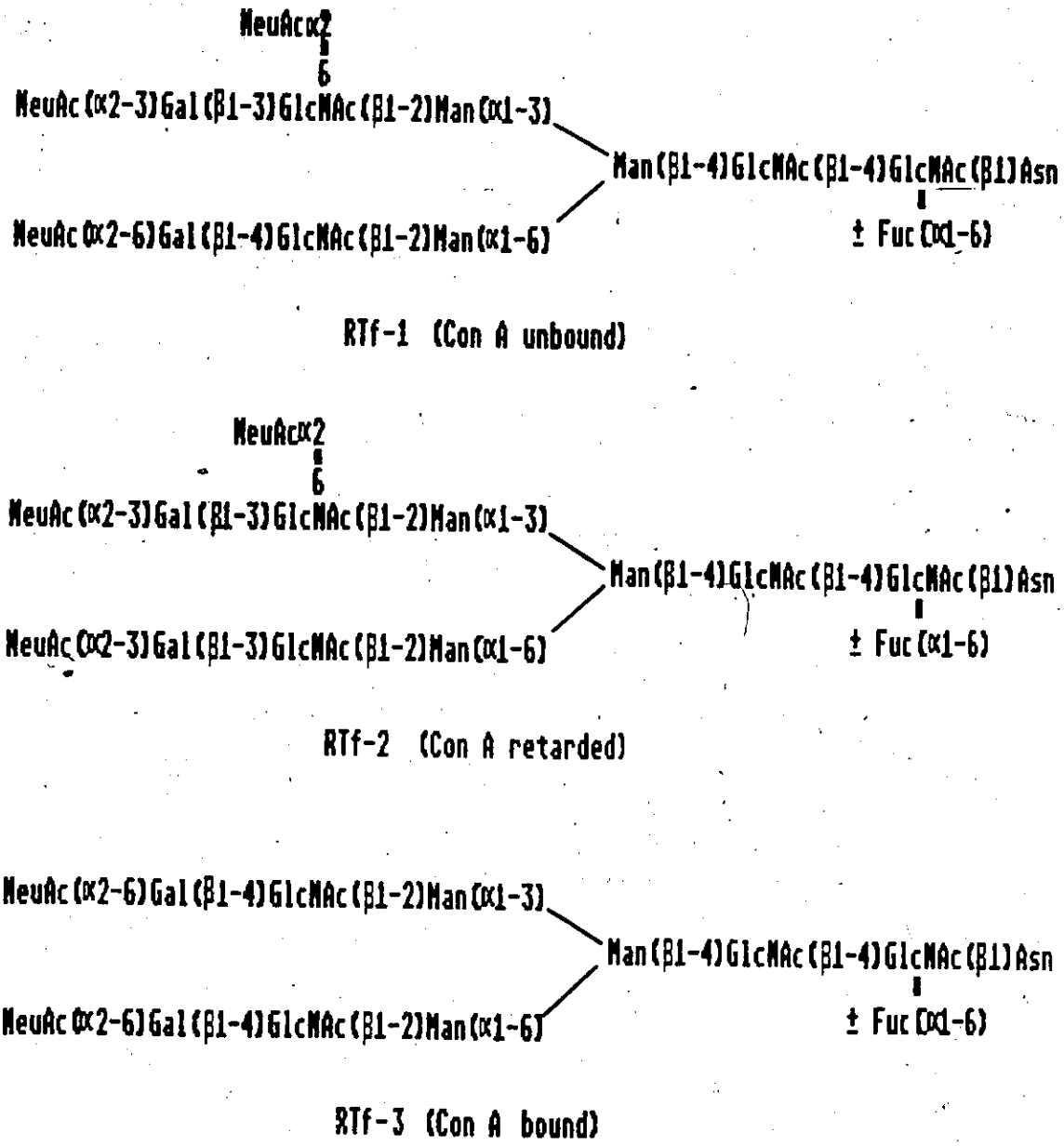

NeuAcα2
6
NeuAc(α2-3)Gal(β1-3)GlcNAc(β1-2)Man(α1-3)
Man(β1-4)GlcNAc(β1-4)GlcNAc(β1)Asn
NeuAc(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-6)
± Fuc(α1-6)
RTf-1 (Con A unbound)
NeuAcα2
6
NeuAc(α2-3)Gal(β1-3)GlcNAc(β1-2)Man(α1-3)
Man(β1-4)GlcNAc(β1-4)GlcNAc(β1)Asn
NeuAc(α2-3)Gal(β1-3)GlcNAc(β1-2)Man(α1-6)
± Fuc(α1-6)
RTf-2 (Con A retarded)
NeuAc(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-3)
Man(β1-4)GlcNAc(β1-4)GlcNAc(β1)Asn
NeuAc(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-6)
± Fuc(α1-6)
RTf-3 (Con A bound)

FIGURE 2.3

FIGURE 2.3 Carbohydrate structures of Rat Transferrin

Our understanding of the degree of microheterogeneity of rat transferrin has been advanced and modified by the above studies in two ways. First, evidence was obtained for the existence of a third component which was distinguishable by its retarded passage through con A-Sepharose. Its consistent presence in the chromatograms (Table 2.1) in spite of the finding that it constituted only about four or five percent of the total circulating transferrin, lent support to the view that it represented a different transferrin molecule with respect to glycan structure and indeed, the structural analysis by Dr. Spik and her colleagues proved this to be correct (see Figure 2.3). Clearly, Schreiber and his coworkers (1979) eluted their con A column too early with methyl glucopyranoside to have discerned the presence of this form of transferrin. The three con A-derived subforms of rat transferrin will subsequently be referred to as RTf-1 (material unhindered by con A), RTf-2 (retarded by con A) and RTf-3 (bound by con A).

Second, the present studies have shown the degree and specificity of fucosylation of serum transferrin. It was demonstrated that a significant portion (20-28%) of the rat transferrin molecules are fucosylated. This conclusion, was firmly supported by the results from the structural analysis provided by Dr. G. Spik and her coworkers. Fucose is a known constituent of lactoferrin

glycans (Prieels et al., 1978; Spik et al., 1982) and of the transferrins investigated so far, the porcine protein is the only other one to have been reported to contain this monosaccharide (Hudson et al., 1973; Graham and Williams, 1975). The two heterogeneities (con A- and lentil-based) are therefore independent of each other. The implication, is that the single glycan present in rat transferrin may assume (at least) six different forms with respect to glycosylation.

An issue which arises from these observations on microheterogeneity is whether the single glycan of rat transferrin is always attached to the same asparagine site. Thus far it has been determined that transferrins from various species have similar amino acid composition (Hudson et al., 1973). Two asparagine sites (ASN 413 and 611) are glycosylated in human transferrin (MacGillivray et al., 1983). The ASN 611 site is not peptide-bonded to a tyrosine and therefore a tryptic fragment containing this glycan cannot be radioiodinated however, the ASN 413 site of glycosylation in human transferrin is peptide-bonded to a tyrosine which can be radioiodinated. The single site of glycosylation in bovine transferrin is also peptide-bonded to a tyrosine which can be iodinated (Graham and Williams, 1975). Recent work on rat transferrin (Regoeczi et al., 1987) demonstrated that glycosylated tryptic fragments of all three con A

subforms were able to be iodinated. By implication, these findings of Regoeczi et al. suggest that the site of glycosylation in rat transferrin might be the same for all subforms. However, since the primary structure of rat transferrin is only partially known (Aldred et al., 1984), the question cannot be answered unequivocally at present.

Given the above understanding of the glycan structures of rat transferrin it is now appropriate to ask what impact these differences in carbohydrate structure have on the biological function of transferrin. This issue is dealt with in detail in the next chapter.

CHAPTER 3

CONSEQUENCES OF THE GLYCAN MICROHETEROGENEITY OF RAT TRANSFERRIN *IN VIVO* AND *IN VITRO*

3.1 Introduction

It has been known since 1961 that rat transferrin is composed of two "populations" (the so-called "fast" and "slow" transferrins; Gordon and Louis, 1963) which are separable by starch gel electrophoresis (Beaton et al., 1961), anion exchange chromatography (Gordon and Lewis, 1963; Schreiber et al., 1979) or concanavalin A Sepharose (con A) chromatography (Schreiber et al., 1979). However, it had not been until the recent structural analysis performed by Spik et al. (see Chapter 2) that the basis for the heterogeneity could be attributed to variations in the structure of a single glycan on the transferrin molecule. The four subforms identified by sequential chromatography on DEAE-cellulose and con A have been designated RTf-1, -2, -3, and -4 as indicated in Chapter 2. It is clear that RTf-4 differs from RTf-3 by the presence of two additional negative charges, however to date its structure has as yet to be elucidated. Therefore, it will not be discussed further.

Thus far, little work has been done to advance the understanding of the possible functional role played

by carbohydrate microheterogeneity in glycoproteins. In fact, the kinds of observations most often reported tend to lend weight to the view that perhaps the carbohydrates perform more general roles including protection from proteases and stabilization of tertiary structure (Berger et al., 1982; Olden et al., 1985) or perhaps to fulfill more of "luxury" functions of cell differentiation (West, 1986). With respect to plasma glycoproteins, only the two subforms of human alpha-1-protease inhibitor (one bearing three biantennary and the other two biantennary and one triantennary glycan (Vaughan and Carrell, 1981)) and rat transferrin (Gordon, 1962; and Heubers et al., 1981) have been investigated with respect to the significance of glycan microheterogeneity for metabolic behaviour. Short-term studies in rats with alpha-1-protease inhibitor demonstrated that the form bearing 2 bi- and 1 triantennary glycan exhibited a shorter half-life than the other type (Vaughan, 1982). Vaughan's results support the proposition that glycan micro-heterogeneity results in functional differences.

Both Gordon and Heubers et al. were looking at functional aspects of microheterogeneity when they examined fast and slow rat transferrin <u>in vivo</u> and in the perfused liver (Gordon). Gordon (1962) reported that the catabolic half-lives were not significantly different nor

was the release of acid soluble radioiodine into the circulation of the perfused liver. The results of Heubers et al. (1981) indicated that the plasma disappearance of iron from the slow and fast forms of transferrin was indistinguishable. However, it is to be remembered that these investigators did not appreciate the degree of microheterogeneity of rat transferrin now known to exist. Indeed, subtle differences may have been missed, or the techniques used not appropriate to measure possible differences. For example, Gordon (1962) compared the half-lives of the two proteins, with one of each in separate animals. Studies on catabolism to be reported below made it patently clear that animal-to-animal variation in catabolic rates can vary significantly and that meaningful results can only be obtained if half-lives are compared in pairs in the same animal.

Therefore, given that the structures of the glycan on RTf-1, -2 and -3 are known and that these transferrins can be obtained to a high degree of purity, it seemed necessary to look at the possible impact of this microheterogeneity in a number of functional settings. As was stated by Aisen (1980) "...it may be that the carbohydrate of transferrin has a recognition function of non-erythroid cells but there is no evidence for this...". The results of these studies provide such evidence.

3.2 Experimental Procedures

3.2.1 Materials

Chromatographic and radioactive materials as in Chapter 2. Neuraminidase (ex Vibrio cholerae) was purchased through GIBCO (Grand Island N.Y.) and collagenase Type IV was from Sigma. Antiserum to rat transferrin was obtained from Cappel Laboratories (West Chester, PA) and monospecific antiserum to rat hemopexin was a gift from Dr. W.T. Morgan, Louisiana State University, New Orleans, LA. Bovine serum albumin (BSA)(Cohn Fraction V) was obtained from Boehringer (Mannheim, F.R.G.). All chemicals used were of analytical grade where available.

3.2.2 Preparation of Transferrins

Human transferrin, which was used to determine non-specific binding of rat transferrin in binding assays with reticulocyte ghosts, was prepared as described elsewhere (Regoeczi et al., 1979). The three characterized subforms of rat transferrin type 1 (RTf-1), type 2 (RTf-2), and type 3 (RTf-3) were isolated from pooled rat plasma by Mr. P. Chindemi in the laboratory of Dr. E. Regoeczi according to procedures already described in Chapter 2. Desialylation of these transferrins was performed as follows: Diferric RTf (subform 1, 2, or 3)

(5-10 mg) contained in less than 1 ml was placed in dialysis tubing in addition to 100 units of neuraminidase. The tubing was sealed and placed in 250 ml of a desialylation buffer (composition: 0.05M sodium acetate, pH 5.5 containing 0.15M NaCl and 0.01M $CaCl_2$) and incubated at 37°C. After 24 h a further 100 units of neuraminidase were added and the desialylation buffer changed. After a final 24 h the protein was saturated with iron then the enzyme, non-desialylated and partially desialylated Tf were separated from the asialo Tf by DEAE chromatography by the procedures already described for DEAE chromatography in Chapter 2. The resulting transferrins are referred to as rat asialotransferrin type 1 (RAsTf-1), type 2 (RAsTf-2), and type 3 (RAsTf-3).

3.2.3 Radiolabelling Procedures

Proteins were radioiodinated using tetrachloroglycoluril as described in Chapter 2. Typically, labelling resulted in the substitution of less than one atom of iodine per molecule of transferrin.

Radiolabelling with $^{59}FeCl_3$ was performed as follows: The particular sialylated or asialotransferrin subform (10 mg) was made to be apo by equilibrium dialysis against 4 x 500 ml of 0.5M citric acid pH 5.5 at 4°C for 24 h. This was followed by dialysis against 4

x 250 ml of 0.1M Tris-HCl pH 7.8 at 4°C for 18 h. From this, 2 mg of the apo protein was adjusted to pH 7.5 with 1M $NaHCO_3$ pH 7.3. To this was added a saturating amount of $^{59}FeCl_3$ which was complexed with a 20- to 30-fold molar excess of tri-sodium citrate. The mixture was allowed to incubate for 4 h , or longer, at 37°C. Radioiron labelled Tf was separated from free radioiron by gel filtration on a column (1.5x 15 cm) of Sephadex G-50 (Fine). It was equilibrated with: a) 5mM Tris-HCl, pH 7.4 containing 0.15M NaCl for radioiron Tfs destined for in vivo experiments and b) phosphate buffered saline (PBS) for experiments in vitro (composition of PBS: per litre - 8.0 g NaCl; 0.2 g KCl; 0.2 g KH_2PO_4; and 1.15 g of anhydrous Na_2HPO_4). 20 min before loading the Tf on the column, 20 ul of ^{59}Fe-citrate (2 mg/ml, pH 7.0) was added to the incubation mixture. The degree of iron saturation was calculated from the specific activity of the $^{59}FeCl_3$ and the activity of the radiolabelled transferrin. Greater than 90% of the sites were saturated in all Tf prepared this way.

3.2.4 Experiments In Vivo

Female Sprague-Dawley rats (180-250 g) which received a standard diet (Purina rat chow) and water ad libitum were used throughout these experiments. The work

concerning catabolism and plasma clearance of lentil-positive and lentil-negative transferrins was performed by Mr. P. Chindemi.

I. Catabolic Studies

The drinking water of animals used in these studies was supplemented with NaI (0.0005% w/v) which was started 48 h beforehand and continued throughout. The catabolic half-lives of the sialylated subforms of rat Tf and their desialylated counterparts as well as the lentil-positive and lentil-negative forms of RTf-3 were studied in pairs. Each pair was differentially radioiodinated and administered to individual animals.

During the course of these experiments, the radiolabels were reversed with respect to each protein pair. In each case, there were no observable differences in measurements of the radioactive decay rates dependent on the particular isotope of radioiodine. Method of iodination was also found to not be a relevant factor (the lactoperoxidase and iodine monochloride techniques were compared with the results from tetrachloroglycoluril iodinated proteins.)

In order to discover if any differences in the half-lives of the proteins were due to subtle differences in the degree of iodination a study of the effects of increasing iodination was conducted. RTf-3 only, was

used and in each case an increased number of iodine substitutions (protein:iodine ratios; 1:1.37, 1:2.83, 1:7.13, and 1:11.07) with both ^{125}I and ^{131}I was performed.

Once the animals had been administered their dose of proteins, total-body radiation measurements were begun with the rat placed in a small-animal counting cylinder which rotated on a platform at a fixed distance from a 3 in. NaI (TI) detector (Regoeczi et al., 1975). Measurements lasted for 1-4 min and were performed twice at each time period. During the time between measurement the animals were housed as was routine, however their bedding was changed every 24h. Measurements of non-injected animals housed with the injected animals demonstrated that there were no significant contributions of radioactivity to the total-body measurements which were due to accumulation of free radioiodide (from the animal's urine) on the animal's exterior.

Catabolic rates were calculated from single exponential sections of the slopes obtained from the data.

II. Functional Studies

The plasma clearance of ^{59}Fe from the three sialylated subforms and the lentil-positive and lentil-negative subforms of RTf-3 was performed by administering

a dose of 90-160 ug of each protein per 100 g via a tail vein. Mr. P. Chindemi performed the work with RTf-2 and part with RTf-3. Venous blood samples were taken from a tail vein (approximately 0.3 ml each anticoagulated with dry heparin) at 5 min then at 15 min intervals over 2 h. Plasma samples (50 ul) were prepared in duplicate to a final volume of 2 ml with 0.15M NaCl.

Delivery of ^{59}Fe from the sialylated and desialylated versions of the three subforms, to the liver was quantified by the following procedure: A dose of transferrin (comparable to that used to measure plasma clearance) was administered by tail vein. Before injection a subsample of each dose solution (50-100 ul) by weight was taken and diluted to exactly 50 ml in a volumetric flask with 0.05% SDS in 0.15M NaCl. From this 2 ml aliquots were counted in duplicate. The remaining dose volume was injected and its volume determined by difference in syringe weight before and after administration. The animals were sacrificed at 30, 60 or 90 minutes. The liver was removed and washed (cold distilled water) then homogenized in 42 ml of a solution containing 32 mg/ml of ^{59}Fe in the form of $FeCl_3$ in citrated pH 7.0 and 0.15M NaCl. 8 ml of 2-octanol was added as an anti-foaming agent. The final volume of the homogenate was measured and 2 ml aliquots were taken in

duplicate for counting. ^{125}I-Tf type 3 was included in each dose in order to distinguish between the radioiron activity which was taken into the liver from that trapped in the vasculature of the liver post mortem. Subtraction of the latter value from the former gave a net value of iron accumulation in the liver. It is these values which are reported below.

3.2.5 Binding Studies with Reticulocytes

Reticulocytes were obtained from rats treated with intraperitoneal (i.p.) injections of phenylhydrazine to induce reticulocytosis (Harding et al., 1983). The resulting blood was >80% enriched with reticulocytes. These cells and the red blood cells were separated from the plasma and white blood cells by centrifugation (1,500 RPM at 4°C) and subsequently subjected to hypotonic shock in order to obtain ghosts of the cell membranes (Dodge et al., 1963).

The binding assays were conducted on ice in 50 mM HEPES [4-(2-hyroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.4 containing 1% (w/v) BSA and 5 mM $CaCl_2$ in a final volume of 0.2 ml. Total binding of RTf was measured at different concentrations of the particular radioiodinated RTf subform (3.75-50 ug/ml) which were incubated with ghosts (1.0-1.75 mg of cell protein/ml)for

1 h. A 60-fold excess of unlabelled human diferric Tf was added 15 min before the radioligand in order to measure non-specific binding. All measurements were performed in duplicate. The incubation was stopped by filtration onto glass microfibre filters (Whatman GF/C) followed by two 4 ml washes with ice cold PBS, pH 7.4, containing 0.5% BSA. The data were plotted according to the method of Scatchard (1949) and subjected to linear regression analysis from which apparent dissociation constants (Kd) were calculated.

3.2.6 Studies with Hepatocytes

The interaction and delivery of iron to cells by the different sialylated and desialylated con A subforms of Tf as well as the lentil-positive and lentil-negative subforms of RTf-1 was studied using freshly isolated hepatocytes.

I. Isolation of Hepatocytes

Parenchymal cells were obtained by collagenase digestion of rat liver by a method described by Hatton et al. (1983). However there were a number of modifications. The initial perfusate volume was increased to 600 ml and contained 1000 units (1.6 units/ml) of heparin sodium (Organon, Toronto) and Desferal (deferoxamine mesylate, CIBA) 0.001% w/v. Desferal was also present in

the collagenase and "stir" solutions at the same concentration. After the 10 min period of digestion, the liver was placed onto a watch glass on ice. The encapsulating membranes of the liver's lobes were cut and peeled away (as much as possible) then using a Pasteur pipette a stream of ice cold stir solution (approximately 15 ml in total) was directed at the liver. This proce dure succeeded in significantly freeing hepatocytes. The remaining cells were freed by gentle shaking of the liver.. The initial cell isolate was added to 85 ml of stir solution and allowed to recover at 37°C for 45 min with swirling and aeration (95% O_2/5% CO_2). Subsequent to filtration the parenchymal cells were separated from non-parenchymal cells first by gravimetric sedimentation for 20 min on ice followed by three differential centrifugations (30 s each at 2-4°C and 40 g) in Hanks balanced salt solution (HBSS), pH 7.4 (for HBSS compostion and further details concerning this procedure see appendix CH-3) containing 1% BSA. This solution is hereinafter referred to as the suspension buffer. Cells prepared in this way were determined to be greater than 90% viable by trypan blue exclusion. Contamination by non-parenchymal cells was less than 2%.

II. Protein Association and Iron Uptake by Hepatocytes

Freshly isolated hepatocytes were kept on ice until they were warmed to 37°C during a 10-min pre-

incubation period in a shaking water bath. The "dose" solutions which contained suspension buffer and the particular ^{59}Fe-labelled transferrin and its radioiodinated counterpart or differently iodinated protein pairs, depending on the study, were added to the cells to give a final volume of 3.4 to 5.0 ml. Before addition to the cells, the quantity of material added in each dose solution was evaluated by measuring the radioactivity of three 10 ul aliquots.

Seconds after addition of the dose, 0.2 ml subsamples were taken in duplicate and centrifuged in an Eppendorf centrifuge (12 000 g) for 10 s. through 0.2 ml of dibutyl phthalate contained in micro-centrifuge tubes. The tips of the tubes were cut off and counted. Free radioiodide in the medium, which remained on top of the dibutyl phthalate, was determined by precipitation of 0.1 ml of the medium with 0.1 ml 20% (w/v) trichloracetic acid. After mixing and standing overnight in the cold the precipitate was removed by centrifugation and 0.1 ml of the supernatant was counted. All subsequent subsamples taken from the incubation at the relevant times were processed in the same way and final counts taken as those remaining after subtraction of counts from the starting subsample.

3.2.7 Other Techniques

All protein concentrations, other than for Tf, were determined by the method of Lowry (1959). Purity of the transferrins was assessed by polyacrylamide gel electrophoresis and was performed as outlined by Laemlli (1970). Hemopexin and transferrin were quantified by rocket immuno electrophoresis as described by Weeke (1973) (also, see Chapter 5, section 5.2.4.3).

The rate at which the different subforms of transferrin - sialylated and desialylated - bound iron was studied in vitro as follows: The apo forms of the transferrins (1.83-2.94 mg) in 0.1 M Tris-HCl, pH 8.0 and containing 0.188 uM Na_2CO_3 were placed in a quartz cuvette in a Beckman DU-40 spectrophotometer. A saturating amount of iron (2.77 mM $FeCl_3$ and 5.55 mM trisodium citrate) was added to give a final reaction volume of 1 ml. Readings were taken at 465 nm at 15-s. intervals for 20 min. The relative rates of iron release were also measured: that information forms the subject material of Chapter 4.

The saturation of the apo transferrins (2.2-2.4 mg) in 1 ml of the above Tris buffer but containing 25 mM Na_2CO_3 was compared by titration with 2 ul additions of ferric citrate (2.77 mM $FeCl_3$ and 55.5 mM trisodium citrate) until no further changes in absorption

at 465 nm were observed.

Statistical evaluation of the data was by either analysis of the variances (ANOVA) or unpaired Student's "T" test.

3.3 Results

3.3.1 Saturation with Iron

Electrophoresis of the transferrin (Tf) subforms, both sialylated and desialylated, in polyacrylamide gel in the presence of sodium dodecyl sulphate (SDS) and mercaptoethanol revealed single bands which migrated identically and corresponded to 77 000-80 000 Da. The sialylated transferrin preparations subjected to quantitative immunoelectrophoresis against a hemopexin specific antiserum revealed no contamination as assayed to a threshold of 0.01%.

After the addition of a saturating amount of iron to the apo forms, the absorbance at 465 nm of all sialylated versions increased at a rate of 1.0×10^{-3}. min^{-1}. mg^{-1} (range: 0.939-1.071) while the corresponding value for the desialylated Tfs was 1.3×10^{-3}.min^{-1}. mg^{-1} (range: 1.226-1.338). This difference was significant ($p<0.001$). However, the subforms reacted comparably when titrated with increasing amounts of ^{59}Fe (see

Experimental Procedures). The A_{465}/A_{280} ratio of all forms was approximately 0.0463 ± 0.004 (SE).

3.3.2 Catabolic Rates

In order to perform this study each sialylated subform of Tf was paired with the other two; this was also carried out in parallel for the desialylated subforms. Furthermore, each sialo Tf was compared with its asialo counterpart. Preliminary experiments with RTf-3, iodinated with increasing substitutions of iodine, revealed that more than 1 atom of iodine per molecule of Tf could significantly alter the half-life of the Tf molecule (Figure 3.1). Indeed, a characteristic "dog-leg" appeared which reflected the denatured character of a significant fraction of the material assayed. The number of substitutions of iodine for preparations used throughout this study was consistently less than 1 atom per molecule.

The basis of comparison was the difference between half-lives of two subforms of Tf in individual animals as opposed to their half-lives in absolute terms i.e. hours. To do this, the extent to which portions of the same protein, labelled with different isotopes can give discernible results. (Such differences are a measure of the reproducibility of iodination, constancy of the

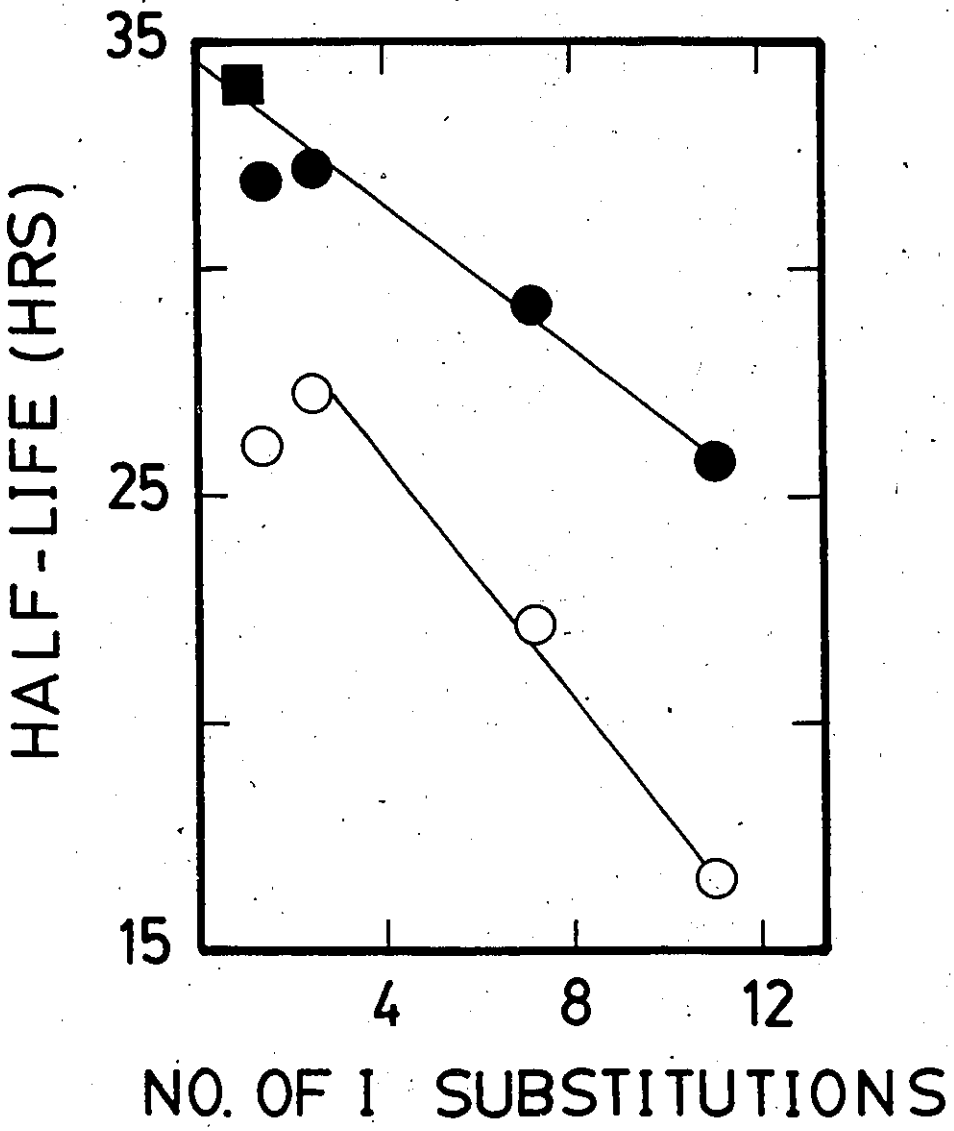


FIGURE 3.1

FIGURE 3.1 Effect of increasing substitution of iodine on the half-life of rat transferrin. RTf-3 was radioiodinated with an average of 1.37, 2.83, 7.13 or 11.07 atoms of iodine per molecule and the catabolic half-life then determined as described in Experimental Procedures (section 3.2.4(I)). Half-life of Tf labelled according to standard procedure (filled square). The radioactive decay curves expressed two distinct half-lives at levels of radioiodination above 1 atom of iodine. The regression line of each section (i.e. 2 lines for each total curve of each protein) was determined. The filled symbols reflect the half-life of the latter part of those curves while the open symbols reflect the early part of the curves. The results represent the means of two determinations.

total-body counter, the correctness of the crossover factor, etc.). Separate study of these values in six animals on three different occasions using three pairs of labelled Tf gave rise to the percentage difference values indicated in Table 3.1 under the heading "Control". The results from thirty-five experiments comparing all Con A distinguished subforms and their desialylated counterparts were analysed similarly in order to arrive at the percentage differences seen in Table 3.1.

Comparison of the mean of the control group with the corresponding mean of particular paired study (e.g. RTf-1/RTf-2) by the students "t" test revealed that the half-life of RTf-1 was significantly longer ($p=0.0036$) than that of RTf-3, while the half-lives of RTf-2/RTf-3 and RTf-1/RTf-2 were not significantly different (see Table 3.1).

With respect to the desialylated proteins, only RAsTf-2 was found to have a significantly shorter ($p<0.00001$) half-life than RAsTf-3. The half-lives of RAsTf-1/RAsTf-3 and RAsTf-1/RAsTf-2 were not significantly different (see Table 3.1).

Results from a last group of experiments indicated

TABLE 3.1 Percentage Differences between the Catabolic Half-lives of Sialylated and Desialylated Con A-distinguished Tf Subforms

*PROTEIN PAIR	Cntl	RTf-1 RTf-2	RTf-2 RTf-3	RTf-1 RTf-3	RAsTf-3 RAsTf-1	RAsTf-1 RAsTf-2	RAsTf-3 RAsTf-2
	0.22	0.47	5.73	9.35	5.14	4.17	12.78
	0.95	4.90	3.01	10.94	5.69	6.84	13.11
	5.68	3.34	4.31	10.86	2.77	6.00	13.29
	3.09	2.28	6.13	8.82	1.49	2.52	8.63
	3.58	0.84	3.07	6.88	2.64	2.76	10.23
	5.76	0.07	6.57	8.90		1.56	
				3.28			
MEAN	3.21	1.98	4.80	8.43	3.55	3.97	11.61
SEM	0.95	0.77	0.64	1.00	0.80	0.85	0.93
P		0.34	0.20	0.0036	0.79	0.56	<0.00001

NOTE: Cntl, control; SEM, standard error of the mean; P is the significance of the "t" value. Percentage differences were obtained as (A-B/A) x 100, where A is longer and B the shorter calculated half-life. See text for further details.

* In each case, the Tf in the numerator had a longer half-life. The range of half-lives was 29.5 h to 44.5 h for sialylated Tfs and 20.4 h to 29.6 h for desialylated Tfs.

that the rate of disappearance of the asialo-proteins was significantly faster than that of the sialylated forms ($p<0.001$).

The range of half-lives in these experiments was 29.5 h to 44.5 h for sialylated transferrins and 20.4 h to 29.6 h for desialylated transferrins. Not surprisingly, ANOVA revealed significant differences between the values obtained for each protein between rats.

Three rats were given a mixed injection of differentially labelled lentil-positive (^{125}I) and lentil-negative (^{131}I) transferrins, both prepared from the con A-binding type, for the comparative study of their catabolism by measurements of total-body radiation. One of these experiments is reproduced in Figure 3.2 for the illustration of the metabolic compatability of both transferrin types. A mean total-body half-life of 31.3 ($\pm$ 0.9 SE) h was calculated for the lentil-binding species, the corresponding value for the nonbinding species being 32.9 ($\pm$ 0.6 SE) hrs. Analysis of the variances showed that this difference was not significant ($F = 1.81$).

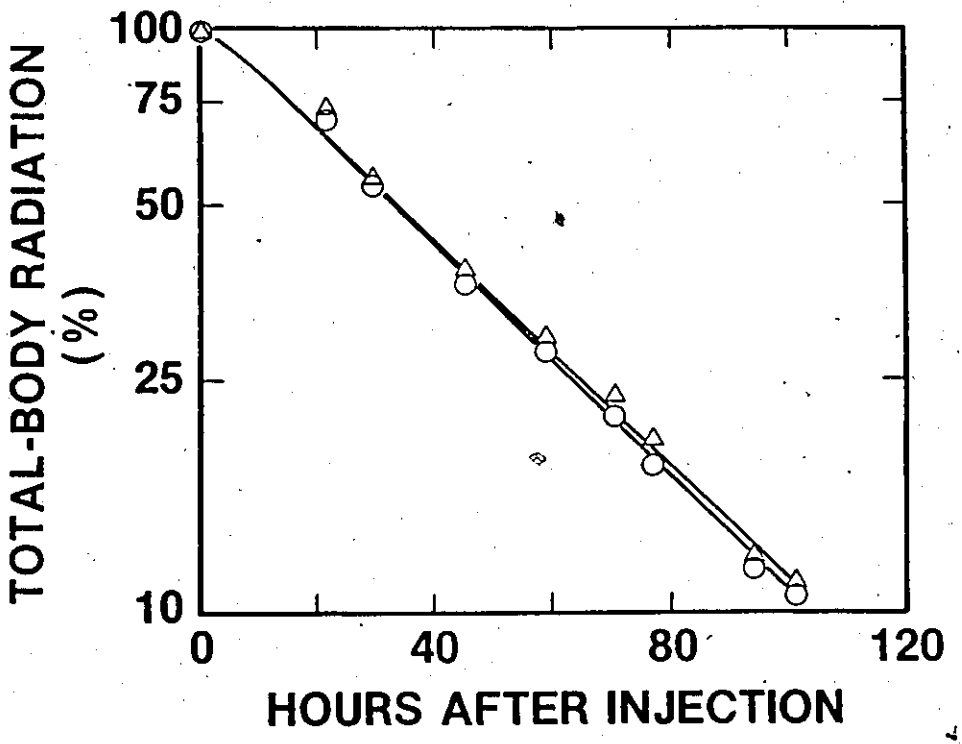

TOTAL-BODY RADIATION (%)
100
75
50
25
10
0
40
80
120
HOURS AFTER INJECTION

FIGURE 3.2

FIGURE 3.2 Total-body radiation of a 240-g rat. Intravenous injection of ^{125}I-labelled lentil-positive (○) and ^{131}I-labelled lentil-negative (△) transferrins. The dose was 10 to 15 ug of each protein. Points represent values expressed as percentages of the initial measurement.

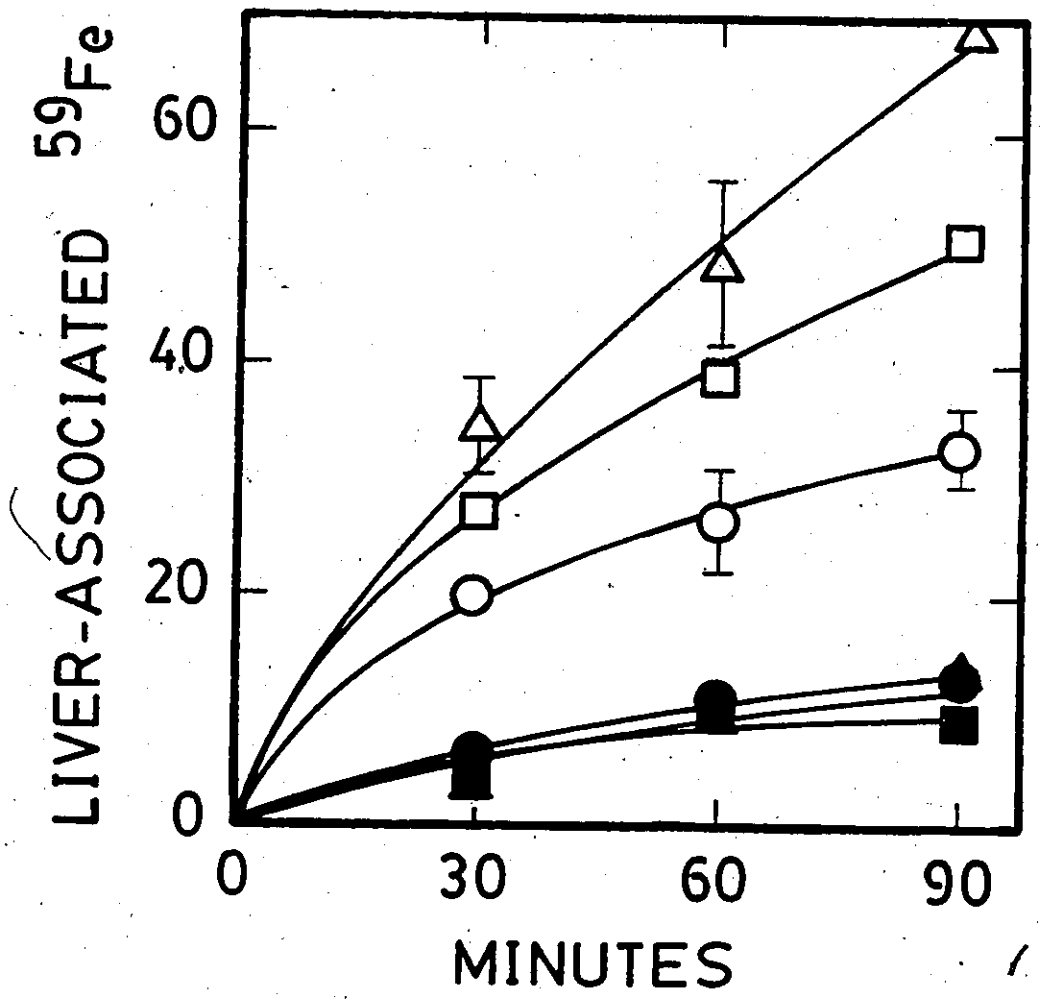


FIGURE 3.3

FIGURE 3.3 Hepatic uptake of iron donated by sialylated (filled symbols) and desialylated (open symbols) subforms of Tf. The net percentage of the dose of radioiron donated to the liver *in vivo* by rat Tf type 1 (triangles), type 2 (squares) and type 3 (circles). Experiments were performed as described in Experimental Procedures (section 3.2.4(II)). Points represent the mean ± SE from duplicate assays for all except RTf-2 which was performed in triplicate with the 90 min time sample repeated six times. Results are expressed as the percentage of the dose administered to the animal. Error bars are shown only when they exceeded the symbol.

3.3.3 Rate of Iron Donation to the Liver and Disappearance from the Plasma

The ability of all subforms of Tf (sialylated and desialylated) to donate iron to the liver *in vivo* was examined and the results are illustrated in Figure 3.3. Three major observations can be made. The first and most noticable is the striking difference in rates of donation between the asialo and sialylated Tfs. Approximately 20%, 40% and 60% more iron was delivered by RAsTf-3, RAsTf-2 and RAsTf-1 respectively, than by their sialylated counterparts after 90 minutes. Second, there is a clear hierarchy of rates of donation among the desialylated versions in the order of: RAsTf-1> RAsTf-2> RAsTf-3. Third, there was effectively no difference in the rates of donation between all three sialylated Tfs each donating approximately 10-15% of the dose by 90 min. Note however, at 90 min, the appearance of somewhat less (not statistically significant) iron from RTf-2 than either RTf-1 or RTf-3 (compare 9.23 $\pm$ 0.93%, RTf-2; with 13.09 $\pm$ 0.08, RTf-1; and 12.61 $\pm$ 1.35, RTf-3 ($\pm$SE)). In 2 h,

15.9 (± 1.7 SE)% of the ^{59}Fe dose was uptaken by the livers of rats which received lentil-negative transferrin, the corresponding value for the lentil-positive preparation being 11.9 (± 1.0 SE)%. This difference is not statistically significant (F=4.41). In view of the similar rates of iron donation by the sialylated and lentil-positive and -negative Tfs to the liver, investigation of the plasma disappearance of iron from these forms of Tf was conducted in order to discern whether any difference in overall iron delivery might be uncovered.

The animals were injected as described in Experimental Procedures (section 3.2.4 (II))and the first 5 min sample was taken to represent 100% of the activity initially present in the plasma. Subsequent samples were compared to this value and expressed as percentage remaining in the plasma. Regression analysis of the logarithmic values of these percentages and the corresponding time values yielded mean half-lives of 43.9 ± 4.49 h (SE) (range: 38.47-52.75 h, N=3) for RTf-1, 62.9 ± 4.38 h (SE)

(range: 51.92-73.84 h, N=5) for RTf-2 and 53.3 ± 5.43 h (SE) (range: 41.82-63.2, N=4) for RTf-3. Analysis of the variances indicated that there were no significant differences however it should be noted that the apparent order of half-lives was in the order of: RTf-1<RTf-3<RTf-2.

Experiments illustrating the plasma clearance of iron from the lentil-positive and lentil-negative forms in rats are presented in summary in Fig. 3.4. It can be seen that the fractional disappearance rate of ^{59}Fe was virtually identical regardless of the type of transferrin injected. The same was also true for the plasma ^{125}I activity curves of the labelled proteins.

3.3.4 Binding Studies

The relative affinity of each subform for the rat transferrin receptor (TfR) was tested with rat reticulocyte ghosts. The results are presented in Table 3.2 as the apparent dissociation constant and coefficient of variation and of determination for corresponding regression lines. It can be seen that within the range

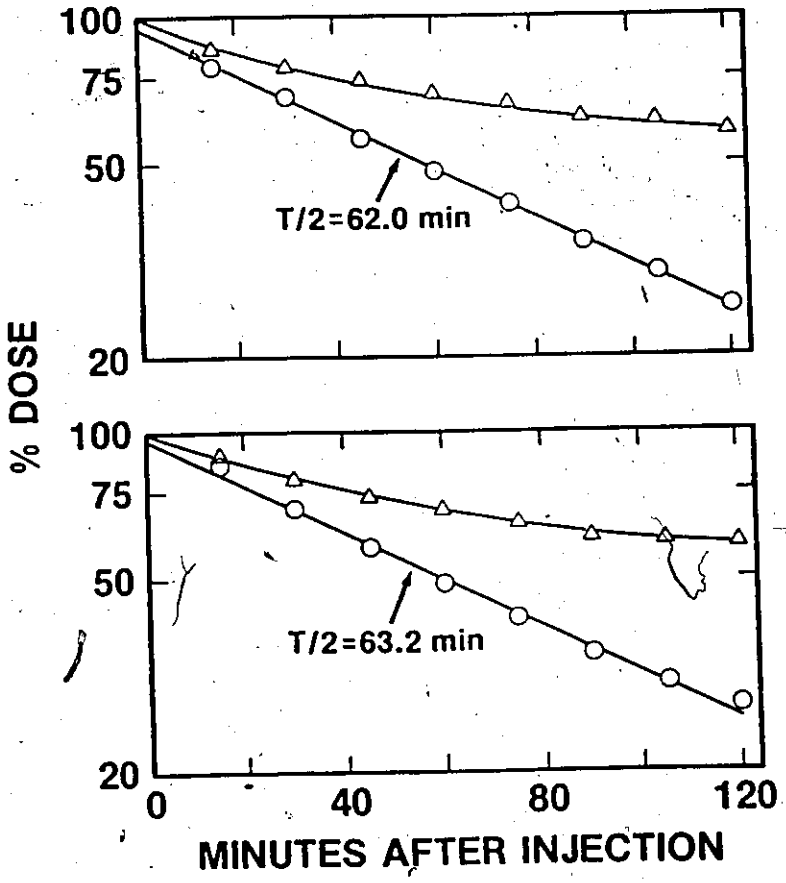

100
75
50
20
T/2=62.0 min
% DOSE
100
75
50
20
T/2=63.2 min
0
40
80
120
MINUTES AFTER INJECTION

FIGURE 3.4

FIGURE 3.4 Plasma clearance of ^{59}Fe from the lentil-nonbinding (upper panel) and lentil-binding (lower panel) forms of iron-saturated rat transferrin of the con A-binding type (RTf-3). Circles denote radioiron and triangles the carrier protein labelled with ^{125}I in the plasma. The doses were 50 to 70 ug of transferrin per 100 g of body weight. Values for upper panel represent the mean of 3 animals and of 6 animals for the lower panel. SEM smaller than symbol. Half-lives were derived by semilogarithmic regression analysis of the group means.

of values for each sialylated subform, overall; there are no significant differences; each of the Tfs, type 1, type 2 and type 3 have similar affinity for the TfR. On the other hand, within the desialylated Tfs RAsTf-2 binds to a significantly greater extent ($p=0.0159$) than either RAsTf-1 or -3. In summary, desialylated Tf type 2 is the only subform different from the rest with respect to its binding affinity, which is higher.

3.3.5 Studies with Hepatocytes

The association of protein and uptake of iron with time, by hepatocytes with respect to the sialylated Tfs was studied simultaneously, on the same preparation of hepatocytes, in the absence and the presence of 1.55 mg/ml diferric RTf (composed of 80% RTf-3, 19% RTf-1 and 1% RTf-2). The results are presented in Figure 3.5. The data in the figure demonstrate the presence of a functionally related difference between the three subforms with regard to both the iron (panels A and C) and protein (panels B and D) association. These are differences which were not distinguishable in vivo (see Figure 3.3).

TABLE 3.2 Scatchard plot analysis of the binding of RTf subforms to reticulocyte ghosts.

RTf Subform	K_D(x10-^{9}M/L)	C.o.V.	r^2
RTf-1	7.65	14.0	0.85
RTf-1	10.54	7.9	0.96
RTf-1	9.30	14.8	0.90
RTf-1	6.31	12.6	0.88
RTf-1	5.62	10.6	0.93
RAsTf-1	6.23	5.3	0.98
RAsTf-1	5.96	10.9	0.91
RTf-2	10.42	15.9	0.84
RTf-2	8.29	13.4	0.87
RTf-2	9.37	10.9	0.89
RTf-2	9.42	14.3	0.83
RAsTf-2	4.54	7.5	0.95
RAsTf-2	3.92	10.4	0.92
RTf-3	8.05	13.8	0.81
RTf-3	6.63	9.0	0.93
RAsTf-3	8.87	13.8	0.84
RAsTf-3	7.03	6.4	0.97
RAsTf-3	8.81	3.6	0.98

C.o.V., coefficient of variation; r^2, coefficient of determination. For further details see text.

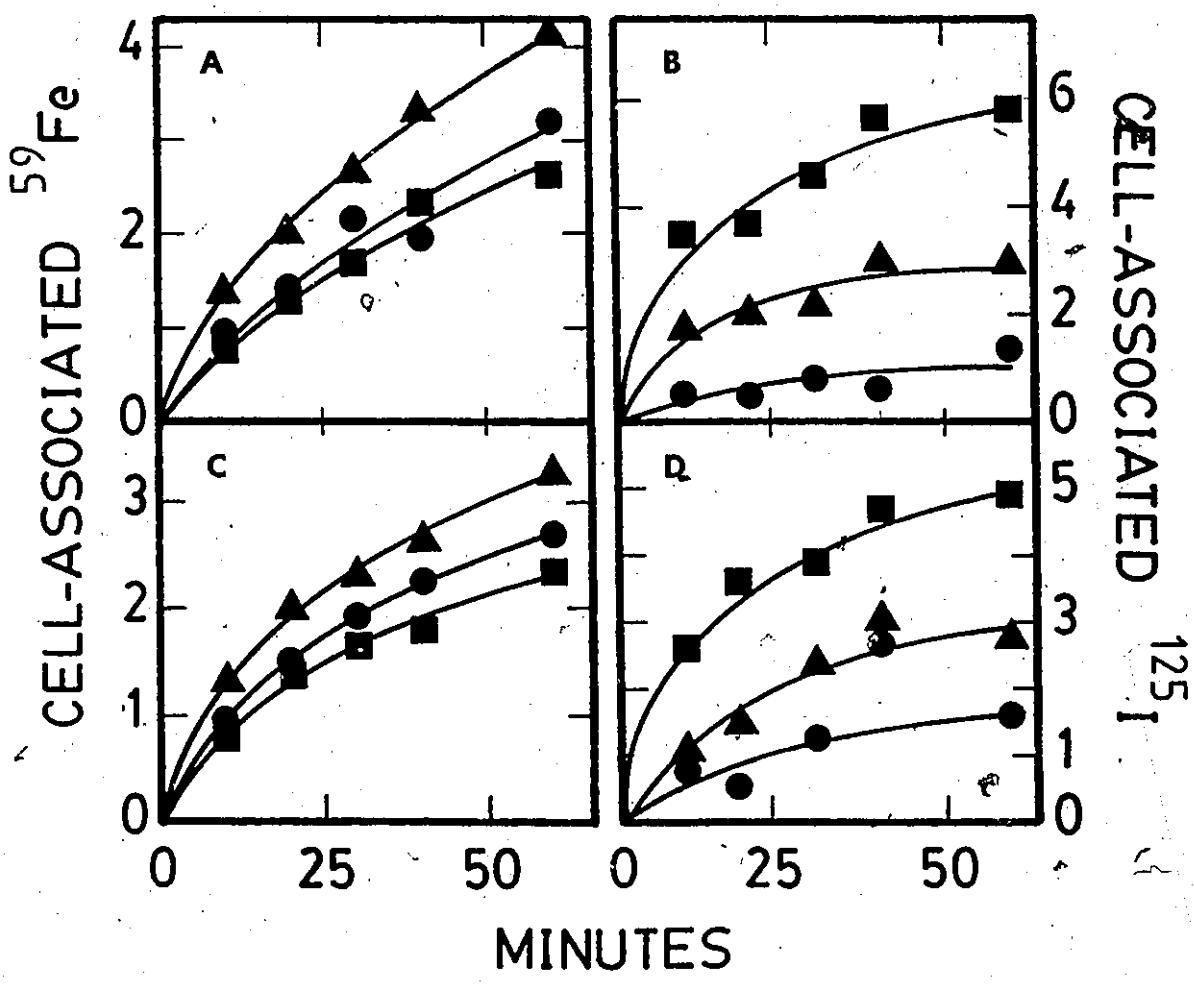

A
B
C
D
CELL-ASSOCIATED ^{59}Fe
CELL-ASSOCIATED ^{125}I
MINUTES
0
25
50
0
2
4
1
3
5
6

FIGURE 3.5

FIGURE 3.5 Cell-associated transferrin and iron uptake by hepatocytes. Uptake of iron was from diferric (radioiron) Tf (50-80 ug/ml of the particular type) and ^{125}I-Tf association of the corresponding Tf type (1.0 ug/ml) by hepatocytes in suspension (9-12 x10^6 cells/ml) from the same liver preparation; in the absence (panels A and B) or the presence (panels C and D) of 1.55 mg/ml rat diferric Tf (mostly type 3). The sialylated Tfs used were type 1 (triangles), type 2 (squares) and type 3 (circles). Experiments were performed as described in Experimental Procedures (section 3.2.6(II)) and the results are expressed as the percentage of the dose added to the cells. Points represent the means ± SE of samples in duplicate.

Aside from the moderate overall depression of protein association and iron uptake, the presence of 1.55 mg/ml of diferric RTf had no effect on the overall trends observed in the absence of excess Tf. Inspection of panels A and C reveals a hierarchy of iron donation with RTf-1 being the best donor followed by RTf-3 then RTf-2 ($p < 0.05$). Comparison of the corresponding panels with regard to protein association demonstrates a different and more pronounced ordering of results. The difference from the pattern observed with respect to iron is that RTf-2 appears to be more rapidly and most highly associated with the hepatocytes. The hierarchy in regard to RTf-1 and -3 is the same as seen with respect to iron delivery.

RTf-1 and RTf-3 are present in abundant concentrations in the circulation of the rat relative to that of RTf-2. Considering the results in Figure 3.5 an examination of the effects of competing concentrations of diferric RTf-1 and -3 on the relationship between RTf-1 and -3 in the respect of protein association and iron uptake by hepatocytes was conducted. Experiments with only RTf-3 and RTf-1 on the same preparation of hepatocytes gave results qualitatively similar to those presented in Figure 3.5 (see Figure 3.6 open symbols). Furthermore, addition of 1.55 mg/ml of diferric RTf-3

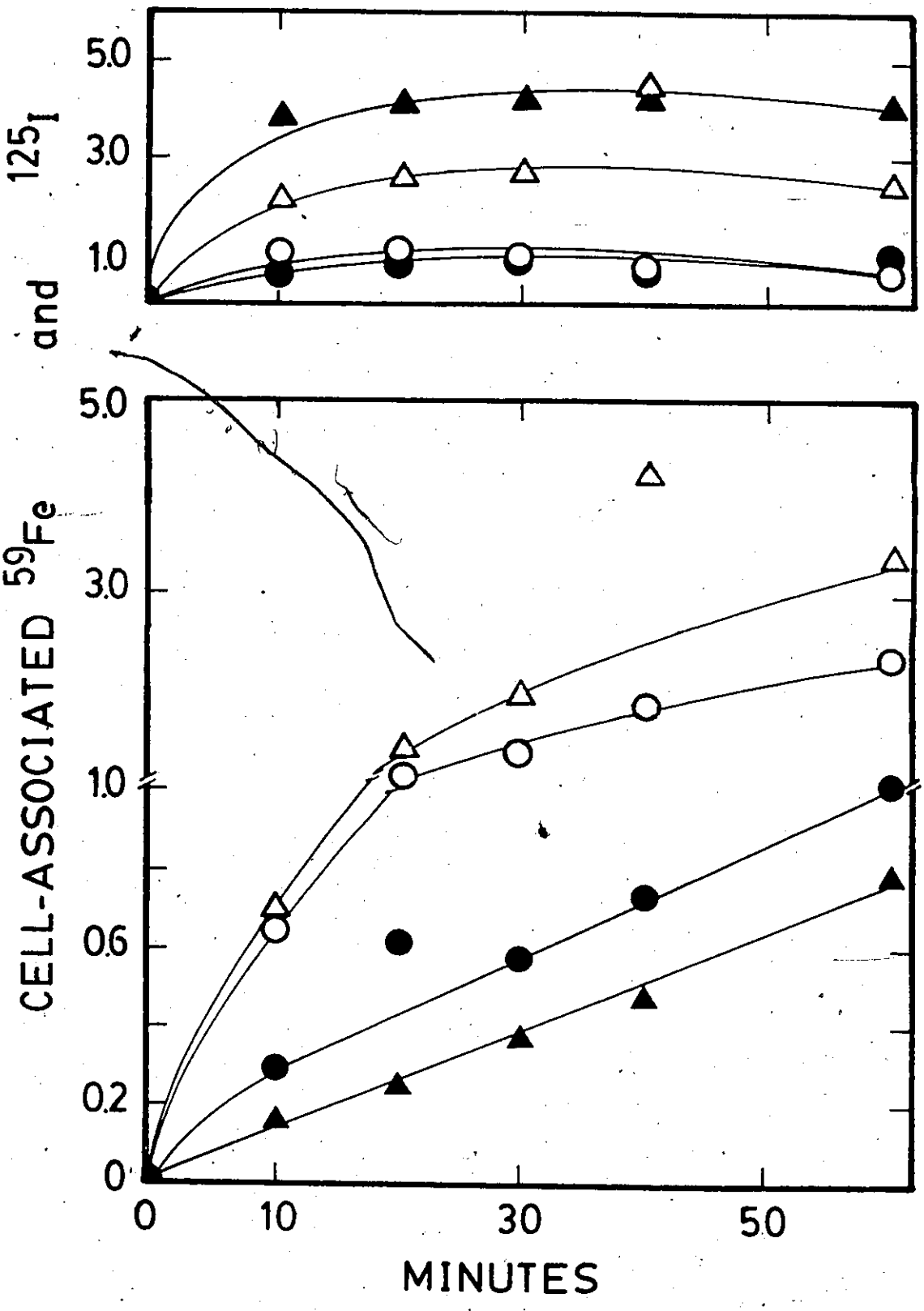

5.0
3.0
1.0
^{125}I and
5.0
3.0
1.0
0.6
0.2
0
CELL-ASSOCIATED ^{59}Fe
0
10
30
50
MINUTES

FIGURE 3.6

FIGURE 3.6 Effect of excess RTf-1 on cell-association and iron uptake by hepatocytes from RTf-1 and RTf-3. Hepatocytes (9-12 x10^6 cells/ml) were incubated in separate cell suspensions with (50-80 ug/ml) of radioiron labelled Tf as either diferric RTf-1 (triangles) or RTf-3 (circles) with 2 ug/ml of the corresponding ^{125}I labelled Tf, in the absence (open symbols) and the presence (closed symbols) of 1.55 mg/ml diferric RTf-1. Samples were taken in duplicate as described in Experimental Procedures (section 3.2.6(II)). The results in the upper panel represent cell-associated protein and results in the lower panel represent iron uptake. Points represent the means $\pm$ SE and are expressed as the percentage of the amount of ligand added.

only resulted in an augmentation of the difference (not shown). However, addition of 1.55 mg/ml of diferric RTf-1 gave results as represented by the closed symbols in Figure 3.6. The presence of the RTf-1 depressed the uptake of iron from both proteins, however, much more so in the case of RTf-1. In effect, the hierarchy of iron donation was reversed, yet, the order of protein association remained unchanged; indeed, the uptake of RTf-1 was augmented.

Uptake into hepatocytes of both ^{125}I-Tf and ^{59}Fe from the lentil-positive (+) and lentil-negative (-) fractions of RTf-1 was compared. Experiments were conducted with cells (20 x 10^6 cells/ml) in suspension at 37°C over a 45-min period (which followed pre-warming for 10 min). Samples were taken in duplicate at 15 min intervals. By the end of 45 min RTf-1(+) and RTf-1(-) had donated 14.83 $\pm$ 0.36 (SE) (results expressed as % of the dose) and 16.59 $\pm$ 0.31 (SE) of radioiron to hepatocytes, respectively. Protein association at the end of the same time period was 1.13 $\pm$ 0.04 (SE) and 1.31 $\pm$ 0.10 (SE) for RTf-1(+) and RTf-1(-), respectively. These values were not significantly different ($F = 4.59$; iron, $F = 2.13$; protein).

3.4 Discussion

The main conclusion to be drawn from the studies presented above is that the glycan microheterogeneity in rat Tf confers attributes which allows one form of Tf to be functionally distinguishable from another. This conclusion rests firmly on the evidence presented *in vivo*, particularly with respect to the differences in rates of catabolism (Table 3.1) and *in vitro*, on the comparison between subforms of iron uptake and protein association in hepatocytes (Figures 3.5 and 3.6). Each aspect of the data as it relates to this interpretation is discussed below.

3.4.1 Rates of Catabolism

Differences in the half-lives of the subforms of transferrin were established here, but not in the earlier work of Gordon (1962) for the reason suggested in the introduction; the variation in measured half-lives of any single protein between animals is large enough to obscure any subtle differences which exist between the proteins. The large range in values for half-lives of the data from these experiments supports this conclusion.

The data concerning catabolism of the subforms of Tf presented in Table 3.1 yields two conclusions. The first conclusion is that the presence of an extra

sialic acid in this particular structural arrangement (compare the structures of RTf-1 and -2 with RTf-3, Chapter 2, section 2.3) is able to extend the life- time of Tf. (The half-life of RTf-2, while not significantly different from RTf-3, was longer, but not as long as that of RTf-1.) The idea of sialic acid playing a role in determining the lifetime of a glycoprotein is not new (Bocci, 1976). With the appreciation of the existence of the asialoglycoprotein receptor (ASGP-R) (Ashwell, 1971) came knowledge that sialic acid prevented recognition of the penultimate galactose moieties; recognition of which would lead to protein destruction. However, the fact that a particular protein with three sialic acids survive longer than the same protein (at present the assumption is that the amino acid backbone of all three subforms is identical) with two, is novel. The results do not allow for the interpretation that the particular location of the sialic acid is a factor, although, neither do they allow for exclusion of this possibility.

If it is assumed that catabolism of a protein occurs predominantly by exposure to cellular interior by usual uptake mechanisms (which presumably increase the probability of encountering the lysosomes) then the finding of longer half-lives for RTf-1 and RTf-2 may be due to a slight, extra, repulsive force which these

Tfs would experience in non-specific adsorptive endocytosis when interacting with the anionic cell surface. The data presented in Table 3.2 demonstrate that affinity of the subforms to the TfR is not affected by the different glycans and therefore this is not a likely factor contributing to the difference in half-lives.

The second conclusion from the catabolic study is that the affinity of desialylated rat Tf for the ASGP-R is rather low as can be inferred from the comparatively much higher plasma disappearance rates of other asialoglycoproteins (Ashwell and Harford, 1982). Thus it is legitimate to ask whether the reduced half-lives of the asialo Tf subforms were due to the ASGP-R or due only to the loss of anionic charge. If the latter were the case, determination of the half-life of RTf without glycan (aglycoTf), which would also be lacking in anionic charge, would likely result in a similar half-life. On the other hand, if ASGP-R recognition was a factor in the half-life of the desialylated Tfs, then the aglycoTf catabolic rate would be somewhat shorter than sialylated Tf but significantly longer than that of the asialo subforms. Indeed, the latter situation appears to be the case. AglycoTf has a slightly longer half-life than asialoTf so that approximately 90% of the change in half-life is referable to loss of charge (Dr. E.Regoeczi, personal communication, 1988).

It can also be concluded that the structural arrangement of the glycan on RTf-2 is sufficiently different from RTf-1 and -3 to allow for the shorter half-life of the desialylated molecule when compared to the desialylated derivatives of the other two proteins (see Table 3.1 - While the half-life of RAsTf-1 was not significantly longer than RAsTf-2, it was longer, although not as long as RAsTf-3). How the structural differences cause this result is not known, however the following is offered as a possible explanation. The Gal β 1-3 linkage on <u>both</u> glycan arms of RTf-2 sets it apart from RTf-1 and 3 (see Chapter 2, Figure 2.3). Considering the importance of spatial arrangement of Gal residues for recognition by the ASGP-R (Schwartz, 1984), it is possible that the presence of two β 1-3 linkages may result in a Gal arrangement with enhanced affinity to the ASGP-R. For example, in a DEAE fraction of HTf which is known to contain a bi- and triantennary glycan , there is separation into two pools (retained and non-retained) based on affinity for Sepharose-immobilized-hepatic lectin (Regoeczi et al., 1979). Because it is also known that the position of these glycans is interchanged in part of this population of HTf molecules (see Chapter 1, Figure 1.3 (3i)) it is possible to conclude that the spatial arrangement of the Gal residues significantly affects the affinity for the lectin.

If this proposal is correct with respect to RTf, then it appears that having only one β1-3 linkage is not enough to enhance affinity given the longer half-lives of RAsTf-1 and -3. In the sialylated state, with respect to catabolism, this appears to be unimportant.

3.4.2 Comparison of Lentil-Positive and -Negative Transferrin

No significant differences could be established in vivo between the lentil-binding and -nonbinding rat transferrin species (Figures 3.2 & 3.4). Neither were there any observable differences in studies with suspended hepatocytes with respect to association of the protein or rates of iron donation. This suggests that they are either fully or nearly fully, functionally equivalent (i.e., any differences between them are smaller than those detectable by the methods employed here). Thus the presence or absence of fucose does not measurably affect the function of RTf with respect to the physiological parameters evaluated here.

3.4.3 Iron Donation to the Liver by Con A Subforms of Transferrin

The higher rate of iron donation to the liver in vivo (Figure 3.3) by the desialyated Tfs is attributable to affinity for the ASGP-R. However, in the light of the

above dicussion concerning affinity to the ASGP-R, the hierarchy of iron donation seen in Figure 3.3 would not be expected. The interaction of RAsTf with the liver is further explored *in vivo* (Chapter 5) and with hepatocytes *in vitro* (Chapter 6). In order to account for the observed differences one explanation may be that different cells of the liver recognize these subforms to different extents. Van Berkel et al. (1987) has succeeded in demonstrating that RAsTf-3 effectively deposits different amounts of iron in endothelial cells and Kupffer cells as well as the parenchymal cells. It is important in this regard to recall the observation of moderate elevation in affinity for the TfR which desialylation confers upon RTf-2 as compared to the other Tfs. Why RAsTf-2 expresses higher affinity for the receptor is not clear. Possibly the glycan of RAsTf-2, only, is able to sufficiently alter the conformation of Tf to give this result. Further differences in affinity for other receptors, for sites of interaction may be relevant in explaining the different rates of iron donation.

A possible factor contributing to the higher rates of iron donation by the desialylated Tfs to the liver may be related to the finding of uniform, higher rates of iron loading of the asialo Tfs (section 3.3.1).

This may be a factor due to potentially different sites of delivery and/or different environments (mechanisms?) of iron release.

3.4.4 The Interaction of the Con A subforms of RTf with Hepatocytes

This study represents the first examination of the interaction of the con A-subforms of transferrin with hepatocytes and the results presented in Figures 3.5 and 3.6 demonstrate the differences which exist in this interaction. At this early stage of understanding of the role of glycan structures in transferrin function it is not possible to explain these findings on the basis of glycan structure. Yet, because the basis of micro-heterogeneity is now attributable to glycan structure, it is tempting to speculate broadly and therefore the following interpretations are suggested, based on the above results, as proposals which relate structure to function. A basic assumption which was made to arrive at these proposals is that every aspect of the three subforms of RTf is identical, e.g. amino acid sequence, except the glycan structures:

1. The NeuAc(α2-3)Gal(β1-3) components of RTf-1 and RTf-2 may be important in enhancing protein/cell-association; and

2. The NeuAc(α2-6)Gal(β1-4) components of RTf-1 and RTf-3 may be important in enhancing iron delivery.

The following arguments form the basis for these proposals.

The only differences in carbohydrate structure which exist between the glycans of the three con A subforms occur with respect to the type of linkages of the terminal sialic acids and the penultimate Gal as well as the presence the third sialic acid on RTf-1 and RTf-2 (see Chapter 2, Figure 2.3). The fact that the glycans of RTf-1 and RTf-2 each have three sialic acids, but that RTf-1 is the best donor of iron while RTf-2 is the poorest donor of iron (see Figure 3.5A and C) suggests that the third sialic acid (compared to RTf-3) is not important with respect to this function. However, RTf-1 and RTf-2 have identical Man(α1-3) branches and both exhibit higher cell-association than RTf-3. Therefore the NeuAc(α 2-3) Gal(β 1-3) linkages may confer protein association with the cell to RTf: It is possible then, that the extra NeuAc(α 2-3) Gal(β 1-3) linkages on RTf-2 result in it having the highest cell-association. The third sialic acid may act to modify this proposed aspect of NeuAc(α 2-3)Gal(β 1-3).

The Man(α 1-6) branches of RTf-1 and RTf-3 are identical, but RTf-1 donates more iron to hepatocytes

than RTf-3, while RTf-3 donates more iron than RTf-2. It is possible that the NeuAc(α 2-6) Gal(β 1-4) linkages confer iron donating abilities (and because of the lack of NeuAc(α 2-3) Gal(β 1-3) linkages, RTf-3 shows the lowest protein association). The enhanced association proposed to be conferred by the NeuAc(α 2-3) Gal(β 1-3) on the Man(α 1-3) arm of RTf-1 added to the proposed quality of the NeuAc(α 2-6) Gal(β 1-4) linkages with respect to iron donation, could be what gives RTf-1 its superior iron donating ability.

Unfortunately the present experiments do not allow for distinguishing between these possibilities. How these structural differences, as subtle as they are, bring about differences in function is not clear. It is interesting and curious to note that in the case of RTf-1 and -3, competition for the iron delivery mechanism is possible with either ligand (see Figure 3.6), but, the protein association is not (at least not as measured by these experimental techniques). In addition, in the case of RTf-2, neither the cell-association nor iron delivery mechanism can be competed for by more RTf-2. How these results might be possible is at present only a matter of speculation.

The experiments <u>in vitro</u> relating to iron uptake and cell-association have revealed subtle differences in

function between the three subforms of RTf. These differences were not measured in the present *in vivo* experiments and most likely this is because these studies were not conducted with the proteins in pairs within the same animal as they were in the catabolic studies. However, it is encouraging to note that the data from the liver uptake and plasma disappearance experiments did suggest the same hierarchy of iron donating ability as *in vitro* (see section 3.3.3). Heubers et al. (1981) conducted experiments to examine the plasma disappearance of iron with differently iron labelled subforms of rat Tf in the same animal and found that there was no measurable difference in the rates. However, they were comparing "slow" Tf with "fast" Tf. "Fast" Tf is now known to be composed of RTf-1 and RTf-2. The iron donating capacity of these Tfs is at odds in that RTf-1 is a superior donor to RTf-2. The net rate of donation of iron by a combination of the two (as would be the case for "fast" Tf) would be less than that for RTf-1 alone. Indeed, the net rate would probably be more similar to that of RTf-3. The conclusion would be the same as that stated by Heubers et al. (1981), i.e., that there is no difference in rates of iron donation.

Two factors which would also tend to obscure measurement of any differences in iron donating capacity

to the liver *in vivo* by the subforms of Tf are 1) the relative preponderance of the different subforms existing in the plasma (the impact of competing amounts of a particular subform was clearly illustrated in Figure 3.6) and 2) the possibility of different cells of the liver interacting with the Tfs distinguishably from the hepatocyte. This latter possibility could be tested by conducting similar kinds of experiments as performed here with the other liver cell types.

Given the highly significant differences in iron uptake from the desialylated Tfs by the liver measured *in vivo* and the inference from the catabolic studies that the desialylated Tfs most likely interact with the ASGP-R, albeit somewhat more weakly than other asialoglycoproteins, the mechanism by which this occurs was investigated in order to shed further light on the interaction of Tf with the liver. The next chapter reports the findings of experiments designed to answer whether the desialylated transferrins release iron at a rate different from the sialylated counterparts while Chapter 5 deals with the possibility of an alternate mechanism of uptake by the liver of desialylated transferrin.

CHAPTER 4

THE RATE OF IRON RELEASE FROM SIALYLATED AND DESIALYLATED TRANSFERRIN

4.1 Introduction

Experiments in vivo demonstrated that each of the desialylated rat transferrin subforms was superior at donating iron to the liver when compared to the sialylated transferrins (see Chapters 3 and 5). Results obtained from work with isolated hepatocytes (see Chapter 6) also demonstrated, that at low concentrations, i.e. < 0.020 mg/ml, desialylated transferrin was a better iron donor than its sialylated counterpart. Although these findings may have been due to the added affinity of the desialylated protein for the Gal/GalNAc receptor (see discussion, Chapters 3 and 5) it is also possible that differences exist between the rates of iron release from the sialylated versus desialylated transferrin and this may account, in part, for the differing rates of uptake.

Fletcher and Huehns (1967) first suggested that a physiological difference may exist between the two iron binding sites of transferrin. After much work though, the question is still not completely decided (see Chapter 1, Appendix CH-1, note 1.11) however the balance of experiments suggest that there is no difference. Yet,

even in the event that there is no physiological difference between iron binding sites, it is quite clear from the above mentioned work that differences in iron release rate may exist on the basis of the presence or absence of sialic acid. It is believed that release of iron from each of the two sites of transferrin proceeds by separate mechanisms (Bates, 1982) when involving attack by a chelate. Indeed, Thompson et al. (1986) have suggested that transferrins without sialic acid may allow attacking chelates easier access for iron release and thereby desialylated transferrin could donate iron more avidly; certainly in vitro it has been demonstrated that the rate of iron loading of asialotransferrins is greater than the sialylated counterparts (Chapter 3).

In order to understand the mechanism of enhanced iron delivery of asialotransferrin more fully, it is important to establish whether or not desialylated transferrin is able to release iron at a faster rate than sialylated transferrin. In addition, it is important to cast light on the possible differences in iron release between the subforms of rat Tf. The experiments reported below served to explore these issues using two different techniques.

4.2 Experimental Procedures

4.2.1 Materials

Sodium cacodylate (Calbiochem) and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) was from Sigma. Tris was purchased from Boehringer Mannheim. 2,3 diphospho-D-glycerate, the pentasodium salt (2,3 DPG) was obtained from Sigma and desferri-oxamine (Desferal) was from CIBA. Trisodium citrate, formic acid, acetic acid and all other chemicals used were of reagent grade and obtained where available.

4.2.2 Preparation of Buffers

In experiments where iron release was mediated by addition of 2,3 DPG ('phosphate-mediated release'), rates were measured at a constant pH of 6.6 which was maintained by either 0.1M sodium cacodylate and 0.15M NaCl, or 0.1M HEPES and 0.15M NaCl. The experiments which involved steps of pH from 8.0 to 5.0 then to 4.2 ('pH-mediated release') were performed with 0.01M Tris/HCl pH 8.0 as the starting buffer then stepped first with an addition of 1.66M acetic acid, then with an addition of 2.6M formic acid.

4.2.3 Proteins

Rat transferrins type 1 and 3 and asialo-transferrin type 3 were prepared as described in Chapters

2 and 3, sections 2.2.2 and 3.2.2., respectively. Human transferrin containing two standard N-linked biantennary glycans per molecule was used here (see Regoeczi et al., 1979).

The apo form of the proteins was obtained by dialysis against 4x500 ml trisodium citrate, pH 4.5, for 24 h followed by dialysis against either 0.05M Tris/HCl, pH 8.0, when used subsequently for iron uptake studies, or one of the two buffers employed in the phosphate-mediated release experiments (HEPES or cacodylate).

The diferric proteins were obtained by two methods. Those proteins used in the pH release experiments were charged with ^{59}Fe using $^{59}FeCl_3$:citrate in a 1:20 molar ratio following the technique described in Chapter 3. Fully saturated transferrins for the phosphate release were prepared by the addition of 25.5 micromoles of ferrous ammonium sulphate and 0.5 micromoles of sodium bicarbonate to each 13 nanomoles of apo transferrin. This solution was allowed to stand overnight at room temperature and was subsequently dialysed against either one of the above mentioned buffers (4x250 ml, overnight). For all diferric transferrins, the degree of saturation was assessed by determining the ratio of absorbances at 465 and 280 nm. Only preparations which exhibited ratios of greater than 0.0414 (i.e. > 90 % saturation) were used.

4.2.4 Measurement of Iron Release from Transferrin

It is widely believed that release of iron to cells requires a low molecular weight intermediate (Glass and Nunez, 1986; Morley and Bezkorovainy, 1985; Morgan et al., 1978). Therefore two approaches, one with diphosphoglycerate as the penetrating anion with Desferal as the chelate and a second with step reduction in pH with citrate as the chelate, have been used here.

A) Phosphate-mediated release: The rate at which iron was released from transferrin was measured by recording the change in absorbance at 295 nm that occured when a diferric transferrin solution was read against a blank solution which contained apo transferrin and the exact same constituents. The solutions, made up to a final volume of 1 ml and contained in a quartz cuvette, consisted of (final concentrations): 2.5 mM Desferal, 2.5 mM 2,3 DPG, 0.1M HEPES or cacodylate, 0.15M NaCl, 0.025 mM transferrin, pH 6.6. The 2,3 DPG was the last addition made to the cuvette, after which the contents were mixed well then readings began and were taken every 2 min for 240 min.

B) pH-mediated release: The rate at which iron was released from diferric transferrin was measured by recording the change in absorbance at 465 nm. Readings

of 0.05 mM transferrin in 0.01M Tris/HCl, 1 uM citrate, pH 8.0 were taken at 5 s intervals for 20 s. Five ul of 1.66M acetic acid were then added, mixed quickly, and readings continued for a further 2 min. Following this, the pH was measured and the procedure repeated save that 2.5 ul of 2.6M formic acid were added instead of the acetic acid. Preliminary spectrophotometric scans (700 nm-260 nm, 500nm/min, minimum absorbance 0.0, maximum absorbance 0.20) of formic acid, acetic acid (0.1M), and tri-sodium citrate or Fe:citrate 1:2 (0.001M) and apo-transferrin (2 mg) revealed no significant absorbance at 465 nm.

All readings were performed in a Beckman DU-40 spectrophotometer at room temperature.

4.2.5 Other Procedures

Purity of the transferrins was assessed by alkaline polyacrylamide gel electrophoresis as described in Chapter 3.

4.2.6 Data Treatment

Readings from the phosphate release experiments were plotted semilogarithmically (log OD versus linear time) which resolved the original nonlinear curve into an initial nonlinear component followed by a linear

component. That non-linear component was resolved by exponential peeling by computer to obtain the rate of release from the first release site (Morgan et al., 1978). Regression lines were fitted to these and the resulting slopes used to estimate the individual half-lives.

Readings from the pH-mediated release experiments were evaluated by determining the time taken, from the first reading after a pH step, to the beginning of constant final steady state readings; This was taken to represent the time to release an atom of iron. The same approach was used to evaluate the second site. The results were expressed as the change in absorbance per unit time per milligram of protein.

Results were compared statisically by the paired students "t" test.

4.3 Results

Rat transferrin type 3 contains only one biantennary glycan per molecule. This situation was exploited in order to assess whether a difference in iron release rates existed between asialo and sialotransferrin. Therefore, transferrins with none, or two sialic acids were prepared. Some of these pure preparations are depicted in Figure 4.1.

DIFFERENTLY SIALYLATED TRANSFERRINS

A B C D E F

FIGURE 4.1

FIGURE 4.1 Rat Tfs bearing different numbers of sialyl residues. Alkaline polyacrylamide gels after electrophoresis of rat transferrins and stained with 4.5% perchloric acid and Coomasie G-250. From left to right: A, completely desialylated Tf-3; B, monosialo-Tf-3; C, sialylated Tf-3; D, mixture of desialylated and mono sialo-Tf-3; E, mixture of mono- and completely sialylated Tf-3 and; F, mixture of completely-, mono-, and asialo-Tf-3. Gels were run top (cathode) to bottom (anode). See text for further details.

4.3.1 Phosphate-mediated Release

Verification of the experimental technique was achieved by comparing the release rates of rat transferrin with those of human transferrin. Morgan et al. (1978) found that human transferrin released its iron at higher rates than rat transferrin (inferred from their Figure 1). Overall, the rates reported here were lower than those reported by Morgan et al. (1978) - these experiments were conducted at pH 6.6 and at room temperature whereas their experiments were performed at 37°C and at pH 6.9-7.1 - yet, it can be seen from the data in Table 4.1 that indeed, human transferrin displayed shorter half-lives of iron release (range at A-site: 198-243 min) than did rat transferrin ($p<0.05$).

The asialo and sialylated transferrins were always tested on the same day. An example of a single "run" with each of these proteins is presented, plotted on a semilogarithmic scale, in Figure 4.2. The results from the experiments performed with these transferrins are summarized in Table 4.1. These data indicated, that asialotransferrin released its iron (range: 320-614 min) from the acid stable, A-site at much the same rate as sialylated transferrin (range: 254-820 min)($p>.05$). The release rates found for the acid labile, B-site were also indistinguishable between the transferrins ($p>.05$).

TABLE 4.1 Summary of Phosphate-Mediated Release Experiments.

Transferrin	n	T1/2 (A-site)	T1/2 (B-site)	RANGE
HTf	3	225.5 ± 14.0	15.8 ± 1.3	13.8 - 18.3
RTf-3	3	465.9 ± 84.9	21.0 ± 0.6	20.2 - 22.1
RAsTf-3	3	465.5 ± 177.8	22.5 ± 0.7	21.6 - 23.8

Note: Half-lives are in minutes. Values are means ± SE. HTf, human transferrin; RTf-3, rat transferrin type 3; RAsTf-3, rat asialotransferrin type 3. For further details see text.

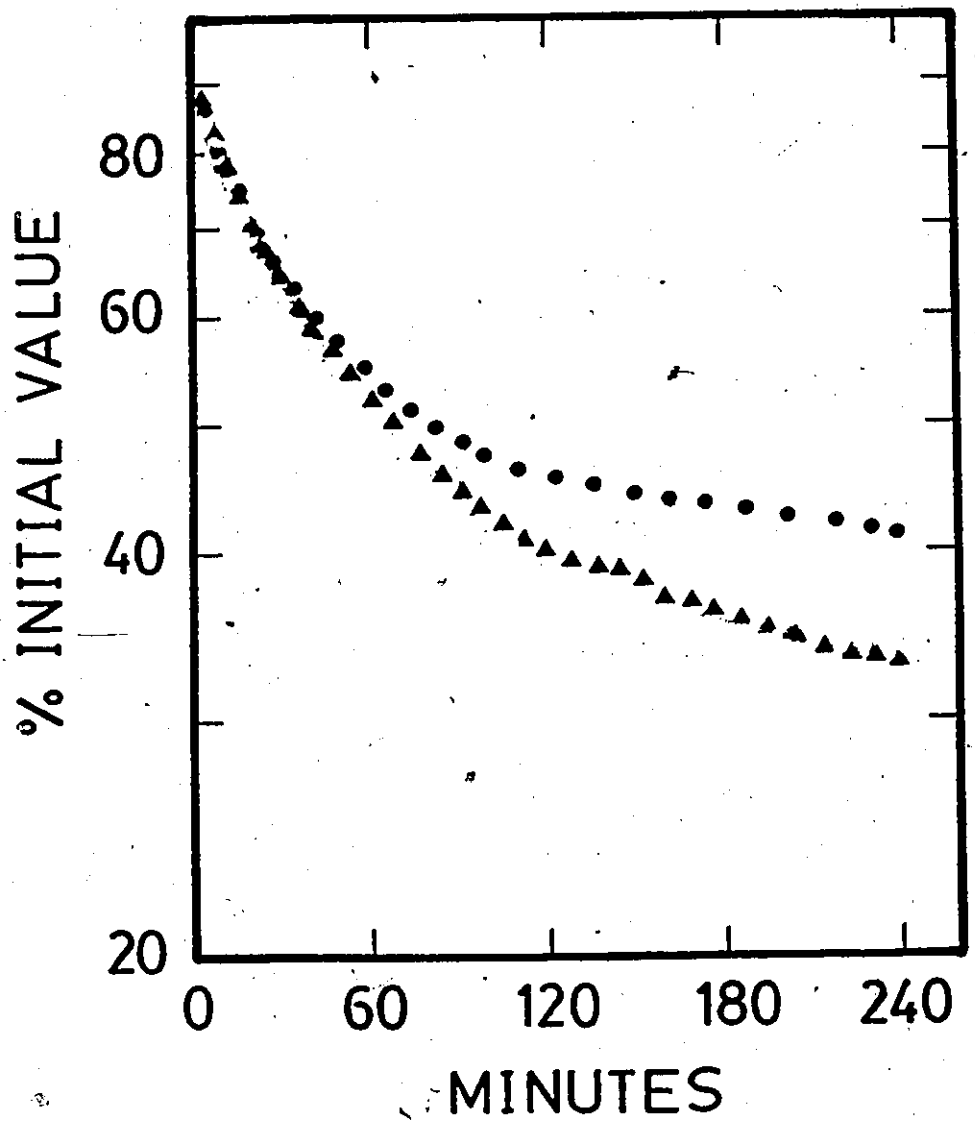


FIGURE 4.2

FIGURE 4.2 Phosphate-mediated release of Fe from asialo and sialylated transferrins. One mg of each of (▲) sialo-Tf-3, and (●) asialo-Tf-3 was added to 25 mM 2,3 DPG, 25 mM Desferal contained in 0.1M HEPES, 0.15M NaCl, pH 6.6 to a final volume of one milliliter. The change in absorbance measured at 295 nm was plotted here as the logarithm of the percentage of the initial reading.

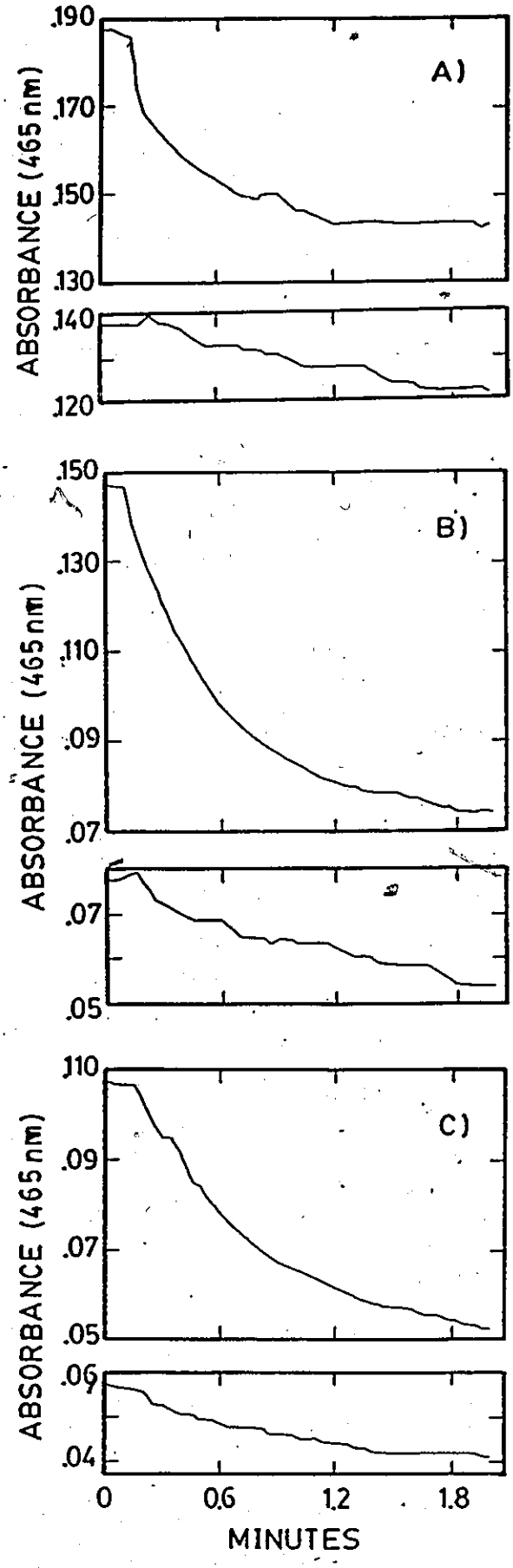

A)
B)
C)
ABSORBANCE (465 nm)
.190
.170
.150
.130
.140
.120
.150
.130
.110
.09
.07
.07
.05
.110
.09
.07
.05
.06
.04
0
0.6
1.2
1.8
MINUTES

FIGURE 4.3

FIGURE 4.3 Single experiments of pH-mediated release of iron from differently sialylated transferrins. A) Sialo-Tf-1 (3.9 mg), B) Sialo-Tf-3 (3.1 mg), and C) Asialo-Tf-3 (2.1 mg). Each protein contained in one milliliter of 0.01M Tris/HCl and 1 uM tri-sodium citrate, pH 8.0. Upper and lower panels, in A, B and C represent the measured change in absorbance at 465 nm after addition of 8.3 nanomoles of acetic acid and 6.5 nanomoles of formic acid, respectively. See text for further details.

4.3.2 Release of Iron Mediated by Sudden Decrease in pH

The possible effect of sialic acid on iron release was further evaluated by using the subform of transferrin containing three sialic acids (type 1, see Chapter 2). An example of an individual 'run' with each transferrin is presented in Figure 4.3. A striking difference between the proteins relates to the final values of absorbance obtained. For both the sialylated and asialotransferrin type 3, the pH step to 5.0 resulted in loss of approximately half of the absorbance - as expected. This was not true for the type 1 protein in spite of the fact that a constant baseline value was achieved by each drop of pH.

The data obtained from all three forms of transferrin are summarized in Table 4.2. The release rate at pH 4.2 (site A) was not found to differ among proteins ($p>0.05$ for each pair). However, the rate at pH 5.0 (site B) suggested that RTf-3 had the fastest release followed by RAsTf-3 ($p<0.05$). The release rate of RTf-1 was found to be significantly different from and slower than RTf-3 and RAsTf-3 at the drop to pH 5.0 ($p<0.01$).

4.4 Discussion

Two conclusions that can be drawn from the results presented here are as follows: 1) potential physiologically important differences possibly exist

TABLE 4.2 Summary of pH-Mediated Release Experiments.

Transferrin	n	Final pH	OD/min/mg
RTf-1	6	5.02 ± 0.1	8.809 ± 0.86
		4.16 ± 0.1	2.907 ± 0.21
RTf-3	5	5.13 ± 0.1	19.085 ± 2.41
		4.19 ± 0.1	3.818 ± 0.45
RAsTf-3	2	5.03 ± 0.0	14.468 ± 0.00
		4.20 ± 0.2	3.979 ± 0.23

Note: OD/min/mg. Values are means ± SE and multiplied by a factor of 1000. RTf-1, rat transferrin type 1; RTf-3, rat transferrin type 3, and; RAsTf-3 rat asialotransferrin type 3. For further details see text.

between transferrins for the first site of iron release (B-site) but sialic acid does not appear to have a straight-forward relationship to this difference and 2) sialic acid does not play a major role in the release rates of iron.

No difference was found in the rate of iron release at the first site in the phosphate-mediated release experiments where the half-life was on the order of 22 min. The cycle time of transferrin through the reticulocyte, however, is on the order of 4-10 min (Nunez and Glass, 1983) wherein a rapid rate of iron release is achieved. In the pH-mediated release experiments which demonstrated rapid release of iron (the first atom was off within two minutes - a physiologically compatible rate), measured speeds were in the order of RTf-3 > RAsTf- 3 >> RTf-1. This order suggests that transferrin with two sialic acids releases the first atom of iron faster than a transferrin with no sialic acids which was faster than transferrin with three. Clearly a straight-forward relationship between sialic acid number and rate of iron release does not exist. The finding of Morgan et al. (1978), that no difference existed between rat transferrin with two sialic acids versus rat transferrin containing three, is reconcilable with the above findings if it is considered that they also used the phosphate release technique which

gives rates lower than those found in a physiological setting.

The findings of the phosphate-mediated release experiments suggest that if there is any difference between RTf-3 and RAsTf-3 (see Table 4.1) it is that RAsTf-3 releases iron more slowly. In terms of overall behaviour this finding agrees with the impression of RAsTf-3 gathered from the pH-mediated release experiments which suggest that RAsTf-3 releases iron more slowly than RTf-3 (particularly from the B-site). This finding is compatible with the results from the iron-loading experiments in vitro which were presented in Chapter 3. There it will be recalled, the "on" rate of iron was uniformly higher for the asialoTfs in comparison with the sialylated Tfs.

The large range in values obtained with the measurements of the second site in phosphate-mediated release experiments makes the true meaning of the result difficult to discern. This range in values may have been due to the three to four hours required to observe the rate of release at this site.

If the release of iron from transferrin within a cell has to occur within 4 - 10 minutes then the rates observed in the pH release experiments shed better light on what might be happening in vivo. No difference in

rates at the second or A-sites was measured over 2 minutes. A subtle difference was found between de-sialylated transferrin and sialylated transferrin at the first or B-site favouring the sialylated transferrin. It is probable however, that the differences measured here do not have a major impact in determining the final outcome with respect to iron donating capacity to the liver. Indeed, work by Morgan et al. (1967) and Aron et al. (1985) has demonstrated no difference in iron donating capacities between human transferrins (sialylated versus desialylated) when incubated with reticulocytes.

A final and important inference may be drawn from the findings of the pH-mediated release study. It is that the release of iron from Tf <u>in vivo</u>, in any cell which takes up Tf, is probably not mediated by a drop in pH alone. Given that a pH of 5.0, at least, is required to dislodge the first atom of iron from rat Tf and that the second atom is quite reluctant to leave, even at pH 4.0 (a value much lower than what has been suggested to occur in endosomes (Geislow and Evans, 1982)), something else must contribute to its release. Therefore it is reasonable to conclude that the change in pH in endosomes occurs (from the cells point of view) mostly to allow dissociation of ligands destined for the lysosomes

and that the reduction in pH is like a bonus which significantly enhances an iron uptake mechanism such as the reductase system (Low et al., 1987).

As suggested in Chapter 3, the Gal/GalNAc receptor available on the surface of liver parenchymal cells may play a key role in the mechanism of enhanced iron delivery observed with asialotransferrin. This possibility will be further investigated *in vivo* in Chapter 5 and *in vitro* in Chapter 6.

CHAPTER 5

PREFERENTIAL HEPATIC UPTAKE OF IRON FROM RAT ASIALOTRANSFERRIN: POSSIBLE ENGAGEMENT OF TWO RECEPTORS

5.1 Introduction

Transferrin (Tf), under physiological conditions, is known to be the most significant supplier of iron to the body tissues. The mechanism which appears to figure highly in mediating this delivery is the diacytotic, pH dependent, receptor-mediated route already outlined in detail in Chapter One.

According to the studies described in Chapter 3, when rat asialotransferrin (RAsTf) is injected into rats it is capable of preferentially donating iron to the liver. Studies performed by Regoeczi et al. (1983, 1984a and b) have demonstrated that the asialotransferrins from man and rabbit as well as rat, when given intravenously to rats, donate their iron preferentially to the liver. How desialylated Tfs give rise to these results is not well understood.

It has been demonstrated that desialylation does not measurably alter the affinity constant of Tf for iron (Jarritt and Charlwood, 1976) which by implication suggests that iron does not dissociate from RAsTf more readily. However, it is clear that the liver contains a receptor site which is specific for galactose (Gal) and N-

acetyl-d-galactosamine (GalNAc) - the Gal/GalNAc-specific lectin - and given that Gal is the penultimate glycosidic residue to sialic acid in rat Tf, the lectin may indeed be playing a role in mediating RAsTf's uptake. In support of this view, Young et al. (1983) have suggested that RAsTf uptake in suspended hepatocytes proceeds either by the lectin and/or the Tf receptor (TfR).

At present it is not clear whether or not the mechanism of "and/or" entry to the hepatocyte in vitro, as postulated by Young et al. (1983), also works in vivo. Aside from the study of Young et al., little work has been performed to understand this phenomenon in general and in particular, no work has been carried out to elucidate the mechanism in vivo.

The results from catabolic studies with RAsTf described in Chapter 3 are difficult to reconcile with the catabolic rates recorded in the in vitro work of Young et al. mentioned above and thus leave open the possibility that the system might not work similarly in vivo. It was with the purpose of examining the particular role that the lectin might play in vivo with regard to the enhanced iron delivery by RAsTf, that the following work was performed.

5.2 Experimental Procedures

5.2.1 Materials

All materials used were as already indicated in Chapters 2 and 3.

5.2.2 Proteins

Human transferrin was prepared as described by Regoeczi et al. (1979). Rat Tf referred to as Rat Tf type 3 (see Chapter 2) was prepared as described earlier (Chapter 2). It was the only form of rat Tf used in these experiments. Desialylation of rat Tf was achieved by the method already described in Chapter 3. Asialo bovine submaxillary mucin was prepared as outlined elsewhere (Regoeczi et al., 1982) but will be presented here briefly. Bovine submaxillary mucin type 1 (obtained from Sigma) (100 mg) was dissolved in 12.8 ml of distilled H_2O. To this was added 3.2 ml of 0.5N H_2SO_4) and the sample was heated at 75°C for one hour in a water bath. The mixture was then cooled to room temperature. The acid was neutralized with approximately 400 ul of 4N NaOH. This procedure results in effectively complete desialylation of the mucin as revealed by electrophoresis (not shown). The preparation was then dialyzed

against 0.15M NaCl, 0.01M Tris, pH 7.4 4x500 ml at 0-4°C for 18 h.

5.2.3 Liver Uptake of Iron

These experiments were performed in vivo with measurements of ^{59}Fe uptake by the liver over time periods lasting for 90 min.

A dose equivalent to 25-75 ug/100 g body weight, of radioiron-labelled RAsTf was mixed with 2-5 ug/100 g body weight of rat ^{125}I-Tf. The final injection volume was on average between 0.3-0.5 ml and contained 0.15 M NaCl with the pH approximately 7.4, the pertinent procedures were performed as described in Chapter 3.

The concentration of circulating diferric Tf was increased by injecting either human diferric Tf intravenously, or iron complexed with nitrilotriacetate (Fe-NTA) intraperitoneally, in a dose of 0.75 mg Fe/100 g of body weight (Yamanoi et al., 1982). Plasma samples, liver homogenates, and other fluid samples were assayed for radioactivity in a Packard model 5986 multichannel analyzer.

5.2.4 Quantification of Total Rat Transferrin and Diferric Tf in Plasma Samples

Quantification of circulating rat Tf with respect to the diferric, monoferric, and apo- states of the protein was performed by a modification of the technique of Makey and Seal (1976) with incorporation of some of the ideas introduced by Leibman and Aisen (1979) as follows.

5.2.4.1 Plasma Samples

Samples from rats were routinely taken from tail bleedings through a 19 gauge needle into 1 ml disposable syringes. The needle, plunger and syringe barrel were all rinsed with a solution of Desferal (7.6 mM), allowed to dry at 80°C, and approximately 0.5 mg of heparin was added before use. After removal of the needle the blood was ejected into an Eppendorf minitube which was centrifuged at 12,000 g for 2 min. The resulting plasma was retained in a separate tube. All tubes were rinsed with the Desferal solution and dried before use. A volume of plasma equal to 26 ul was added to 174 ul of an 8M urea sample buffer which contained: 2.4 g urea; 0.5 ml 1M Tris/borate pH 8.3; 0.6 ml of 5 mg/ml Desferal; 1 g of sucrose and distilled H_2O to a final volume of 5 ml. From this, 30 ul samples were subsequently loaded onto a

6M urea slab gel (see below). A further 20 ul of plasma were diluted in barbital buffer (26 g barbitone-Na; 4.14 g barbituric acid; 112.4 g glycine and 90.4 g Tris made up to 4 litres with a final pH of 8.4-8.5) (1:200) for rocket immunoelectrophoresis (see below).

5.2.4.2 Urea Polyacrylamide Gel Electrophoresis

In order to prevent metal contamination of the plasma samples, Desferal was introduced to all aspects of the procedure. The slab gel (18 x 16 cm x 1.5 mm) containing 1.02 mM Desferal was made 6M with respect to urea in 1M Tris/borate pH 8.3. The stacking gel contained 6M urea and 1.02 mM Desferal while the running buffer contained 6M urea and 0.1 mM Desferal. Subsequent to sample loading, the gel was electrophoresed at 11.25 V/cm for 1 h then for 7 h at 9.4 V/cm at 4°C. Strips to be used for immunoelectrophoresis were cut to be 5.5 mm wide with a double edged tool prepared from sheet metal. Prior to this, the lower 2.5 cm of the slab gel was removed and stained with perchloric-Coomassie G-250 for 20 min in order to visualize the exact location of the "track runs". This was followed by washing in 4 x 100 ml distilled H_2O, then the section was reabutted with the gel. This lower stained portion of each track as well as the individual wells of the stacking gel

provided a guide to ensure reproducibility of cutting in the centre of each "track". Staining of the remaining gel pieces ensured accuracy of the cut. The strips were then placed in 40 ml screw cap bottles and washed for 20 min with 20 ml of barbital buffer in a shaking ice bath. Two further washes were performed with 10% (v/v) glycerol in the buffer. The bottles containing the gel strips were then stored at -40°C or run immediately on immmunoelectrophoresis.

5.2.4.3 Immuno Techniques

The rocket electro-immuno-assay was based on the technique of Weeke (1973). Electrophoresis in gels made to be 1% agarose (w/v)(Seakem MF) in barbital buffer and were 1.5 mm thick on 8x8 cm glass plates was started at 20 V/cm for 1 h then reduced to 12 V/cm for a further 23 h at 4°C. The resulting rockets were visualized by drying and staining in Coomassie G-250. Tracings on tracing paper of the rockets were cut, weighed and compared to standards of known quantities of Tf processed in the same way.

Immunoelectrophoresis of the washed gel strips was performed on 1.5 mm thick 1% agarose gels on 10x8 cm glass plates. The strip was overlaid on a non-antibody gel portion with the dimensions of 10x2 cm. This section was poured first with 3 ml of the 1% agarose. A second

8x10 cm glass plate provided a barrier to the rest of the first plate. The remaining 6x10 cm section was poured with 10 ml of 1% agarose and contained 40 ul of a polyclonal anti-rat Tf antiserum. Electrophoresis at 4°C was started at 20 V/cm for 1 h then continued at 12 V/cm for 23 h. Gels were then dryed and stained as indicated above. Quantification of the resultant diferric Tf curves was performed as described for the rockets above.

5.2.5 Binding of Human and Rat Diferric Transferrins to Reticulocytes and Hepatocytes

5.2.5.1 Preparation of Reticulocytes

Rats were injected with phenylhydrazine-HCl according to the method outlined by Morgan and Baker (1969). Reticulocytes were harvested from the resulting reticulocyte-rich blood by a procedure described by Pan et al. (1983). Dimethyl sulfoxide 10% (v/v), was added to the resulting cell suspension and was divided into 3 ml portions. These were then frozen in liquid N_2 and stored at -40°C.

For a binding assay these cells were thawed on ice (3-6 ml were used depending on the assay). They were then washed once with 50 ml of Hank's balanced salt solution (HBSS) and centrifuged at 2,000 g for 10 min at 4°C. The resulting supernatant was discarded and the

cells were resuspended to an appropriate volume in HBSS.

5.2.5.2 Preparation of Hepatocytes

Hepatocytes were isolated from adult rat livers by perfusion with collagenase as described in Chapter 3 and Appendix CH-3. The viability of hepatocytes was greater than 85% as determined by trypan blue exclusion.

5.2.5.3 The Binding Assays

Binding assays with either freshly isolated hepatocytes or thawed and washed reticulocytes were performed on ice. The incubation medium in all cases contained 150 mM NaCl, 1% BSA, and 1% 1-O-methyl-B-D-galactopyranoside (to block the Gal/GalNAc specific lectin) in 50 mM HEPES, pH 7.4. The final volume was 0.2 ml. A 20-fold excess of nonradioactive human or rat diferric Tf (depending on the particular assay) was added 15 min before the radioligand to measure nonspecific binding. Total and nonspecific binding was measured in triplicate and duplicate, respectively. The assays were terminated after 60 min in the following manner: Hepatocytes were centrifuged through 0.2 ml of dibutyl phthalate at 12,000 g. The tips of the tubes, which contained the cell pellets were cut off and counted in 15x85 mm glass tubes. Reticulocytes were separated from

the incubation medium and free ligand by filtration through Whatman GF/C glass microfiber filters. They were washed with 10 ml of ice-cold HBSS containing 0.5% bovine albumin. The filters were placed in 12x85 mm glass tubes and counted in a Packard model 5986 multichannel analyzer.

5.2.6 Radioiodination and Labelling with ^{59}Fe or ^{55}Fe

Radioiodinations of both human and rat Tfs were carried out using chloroglycoluril as described in Chapter 2. On average, the number of I atoms substituted per molecule of transferrin, using this technique, was calculated to be in the range of 0.14 to 0.70. In a number of binding experiments with human Tf, 6-7 iodinated 3-(4-hydroxyphenyl)propionic acid groups were coupled to each molecule by the procedure developed and described by Bolton and Hunter (1973).

Radioiron and non-radioactive iron were incorporated into the Tfs by the method described in Chapter 3.

5.3 Results

5.3.1 Liver Uptake *In Vivo* of Iron from Rat Diferric Transferrin and Diferric RAsTf Under Physiological Conditions

During initial studies, six rats were maintained under the boarding and nutritional conditions indicated in Chapter 3, section 3.2.4. It was found by rocket immunoelectrophoresis that their plasmas contained 5.0 ± 0.09 (SE) mg Tf/ml. Urea gel electrophoresis and immunoelectrophoresis showed that of this total, 31.3 ± 2.8% (SE) was in the diferric form.

Summarized in Figure 5.1 are the data which represent iron uptake by the liver from a tracer dose (25-75 ug/100 g) of rat diferric Tf or AsTf. As can be inferred from the figure, approximately 10% of the iron from a diferric Tf dose could be found associated with the liver after one hour. By measuring the initial distribution (at 5 min) of radioiodinated Tf in five animals it was possible to estimate the average plasma volume of the rat. The value obtained, 3.1 ± 0.1 ml plasma/100 g (± SE), coupled with the concentration of diferric Tf in the plasma as indicated above, yielded a value of 7.7 ug of iron as diferric Tf per 100 g of body weight. This value taken together with the rate of iron uptake results in a calculation of the average hepatic turnover rate as 0.8 $ug.h^{-1}.100\ g\ body\ wt^{-1}$.

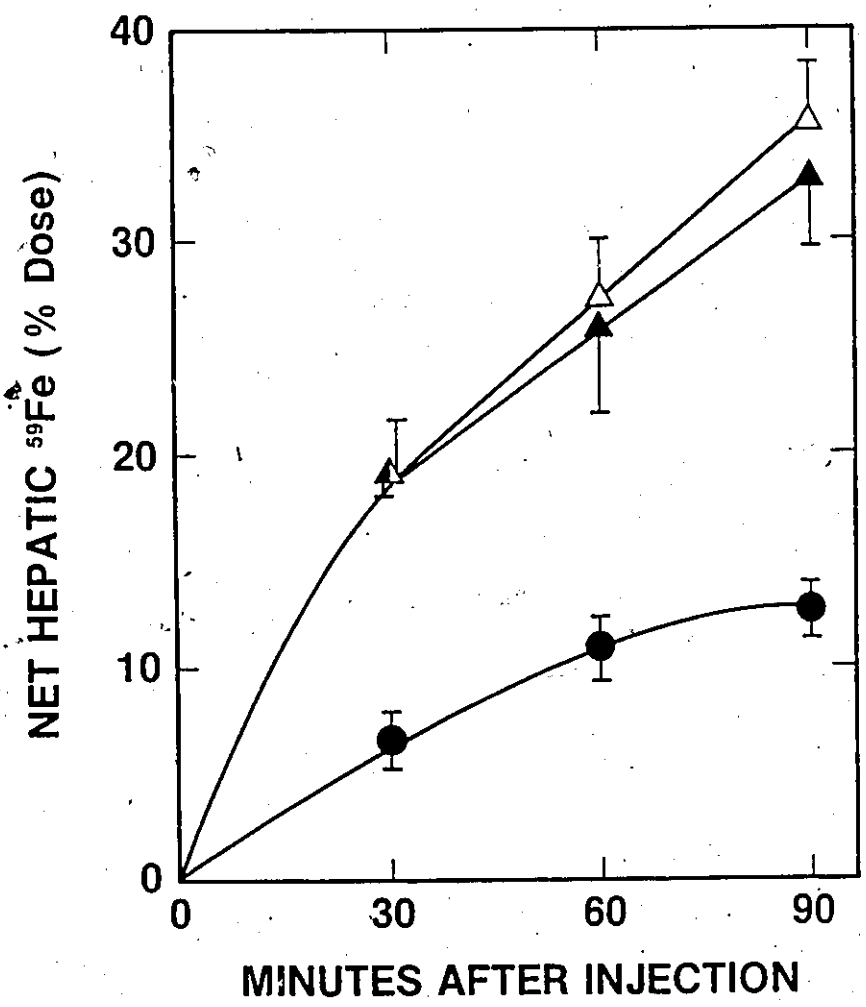

40
30
20
10
0
NET HEPATIC ^{59}Fe (% Dose)
0
30
60
90
MINUTES AFTER INJECTION

FIGURE 5.1

FIGURE 5.1 Uptake of ^{59}Fe from rat diferric Tf (●) and rat diferric AsTf (▲) by the liver in vivo. The uptake of radioiron from RAsTf in animals pretreated Fe-NTA is also shown (△). The proteins (25-75 ug/100 g) were injected intravenously and the Fe-NTA (corresponding to 0.75 mg Fe/100 g) was given intraperitoneally 1 h before the protein. Points indicate the means ± SE from groups of 3 rats each.

The data presented in Figure 5.1 also reveal that diferric RAsTf was a more potent hepatic donor of iron than its sialylated counterpart confirming the findings reported in Chapter 3 (see section 3.3.3). The uptake rates from RAsTf exceeded those from Tf three- to six-fold. The circulating diferric Tf concentration was increased by pretreating the animals with intraperitoneal Fe-NTA (0.75 mg Fe/100 g). In spite of the fact that the Fe-NTA was demonstrated (by urea gel electrophoresis and immunoelectrophoresis, see section 5.2.4) to have quantitatively converted all of the circulating rat Tf to the diferric form, uptake of iron from RAsTf was not altered. Saturation of the plasma Tf with iron from Fe-NTA was also verified by the fact that plasma samples withdrawn from animals up to 3 h after loading with Fe-NTA were unable to bind radioiron *in vitro*.

5.3.2 Reduced Liver Uptake of ^{59}Fe from Rat Diferric AsTf in the Presence of Asialomucin and/or Human Diferric Tf

As can be seen in Figure 5.2, asialomucin (2 mg/100 mg) was able to completely inhibit the enhanced uptake of iron by the liver from rat diferric AsTf. It is salient to note that only excessive iron uptake was inhibited. That is, iron uptake from AsTf still

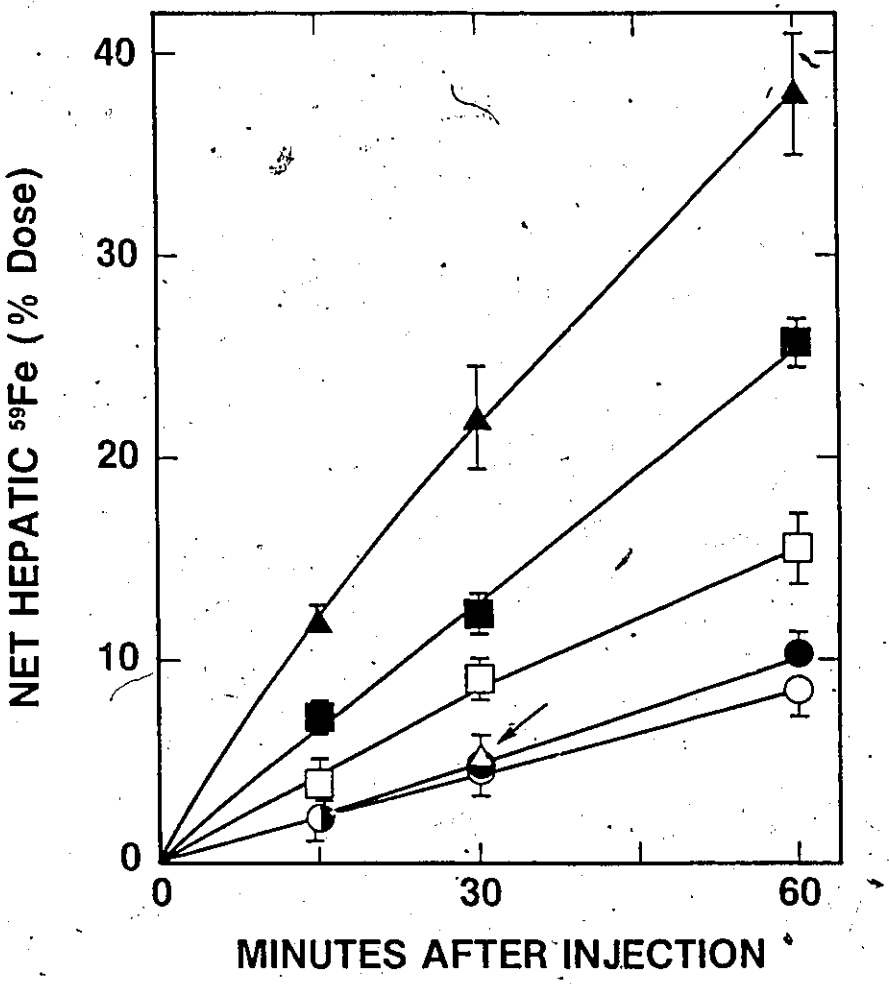

NET HEPATIC ^{59}Fe (% Dose)
40
30
20
10
0
0
30
60
MINUTES AFTER INJECTION

FIGURE 5.2

FIGURE 5.2 Uptake of ^{59}Fe from rat diferric AsTf under various conditions, representing: filled triangles, no treatment; filled squares, human diferric transferrin (10 mg/100 g) given intravenously 2 min before dose; open squares, human diferric transferrin (10 mg/100 g iv) and Fe-nitrilotriacetate (0.75 mg Fe/100 g ip) injected 2 min before dose; open triangle with arrow, human diferric transferrin (23 mg/100 g) intravenously 2 min before dose; open circles, bovine submaxillary asialomucin (2 mg/100 g iv) 2 min before dose; filled circles, control ^{59}Fe-labeled rat diferric transferrin, no treatment. Points are means ± SE from 3-4 rats each.

proceeded at a rate comparable to that from control rat Tf. Human diferric Tf had an effect similar to asialomucin when administered in a dose of 23 mg/ml. It can be seen from close inspection of Figure 5.2 that only a very high dose of human diferric Tf was able to achieve this result; pre-treatment with a dose of 10 mg reduced hepatic accumulation of iron by only approximately one-third while simultaneous intraperitoneal administration of Fe-NTA served to potentiate the effectiveness of human diferric Tf at this concentration. This potentiation effect is probably due to the ability of Fe-NTA to keep human Tf in the diferric state over a longer time period given its ability to donate iron at a higher rate than rat TF (see Regoeczi et al., 1984a).

When both asialomucin (2 mg/100 g) and human diferric Tf (15 mg/100 g and Fe-NTA i.p.) were administered simultaneously, the enhanced iron delivery by rat AsTf was prevented, and once again, the uptake of iron was observed to occur at a rate comparable to that of rat diferric Tf (data not shown).

In control experiments, the combined administration of asialomucin and human diferric Tf had no effect on the rate of iron uptake by the liver from rat diferric Tf. This basic (apparently unalterable) rate is illustrated in Figures 5.1 and 5.2.

5.3.3 Binding of Rat Diferric Tf and Human Diferric Tf by Hepatocytes and Reticulocytes

It was surprising that a 10 mg dose of human diferric Tf was able to reduce the enhanced uptake of iron from RAsTf by the liver when a comparable load of diferric rat Tf (achieved with Fe-NTA) caused no apparent change. (Contrast the results in Figure 5.2 with those in Figure 5.1.) In order to rule out the possibility that this difference in results was brought about through the presence of contaminating human Tf lacking sialic acid residues (Wong and Regoeczi, 1977), the human Tf preparations were electrophoretically monitored and rechromatographed as necessary.

An alternative explanation could be that human diferric Tf has a higher affinity than rat diferric Tf for the rat TfR. Binding experiments conducted with transferrins from both species on rat reticulocytes and rat hepatocytes illustrated that, indeed, this was the case. The results from hepatocytes, depicted in Figure 5.3, demonstrated that the amount of human diferric Tf necessary to inhibit radiolabelled rat Tf by 50% was approximately seven times less than the amount of rat diferric Tf. The same sort of relationship is illustrated in Figure 5.4 for reticulocytes. This implies that the affinity relationship of these two

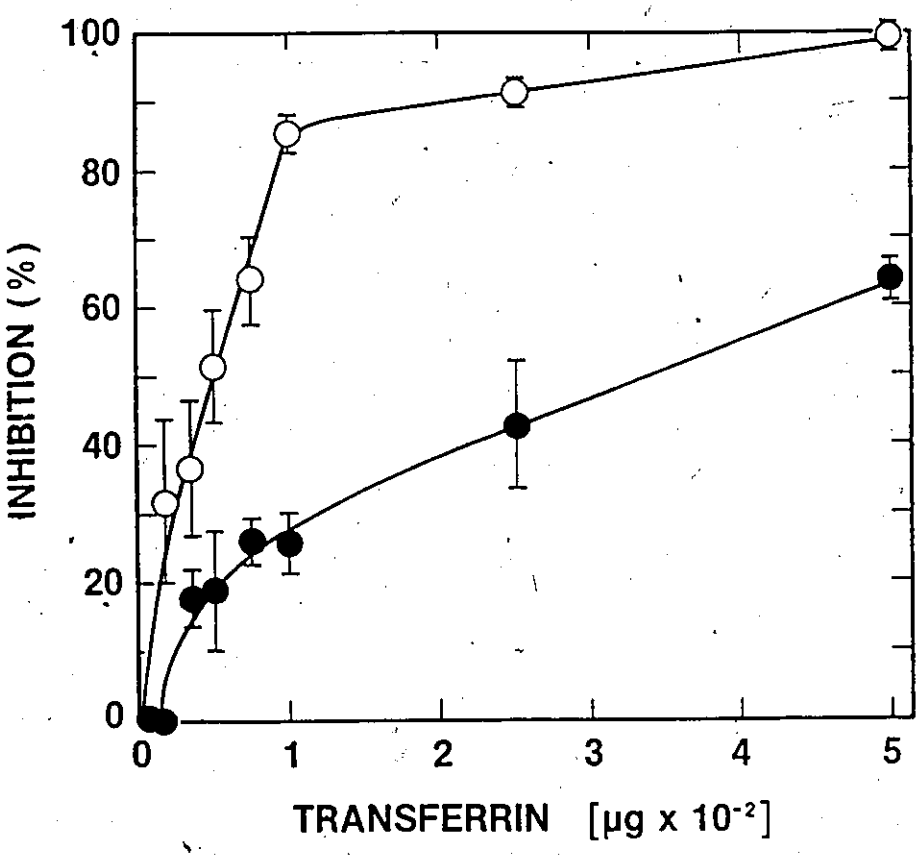


FIGURE 5.3

FIGURE 5.3 Inhibition of binding of rat ^{125}I-diferric transferrin to rat hepatocytes by human diferric transferrin (open symbols) and rat diferric transferrin (closed symbols). Each sample contained 1 x 10^6 cells and 0.5 ug of radioligand. Binding assays were performed as outlined in Experimental Procedures (section 5.2.5.3). Points, representing specific binding, are means ± SE of measurements in triplicate.

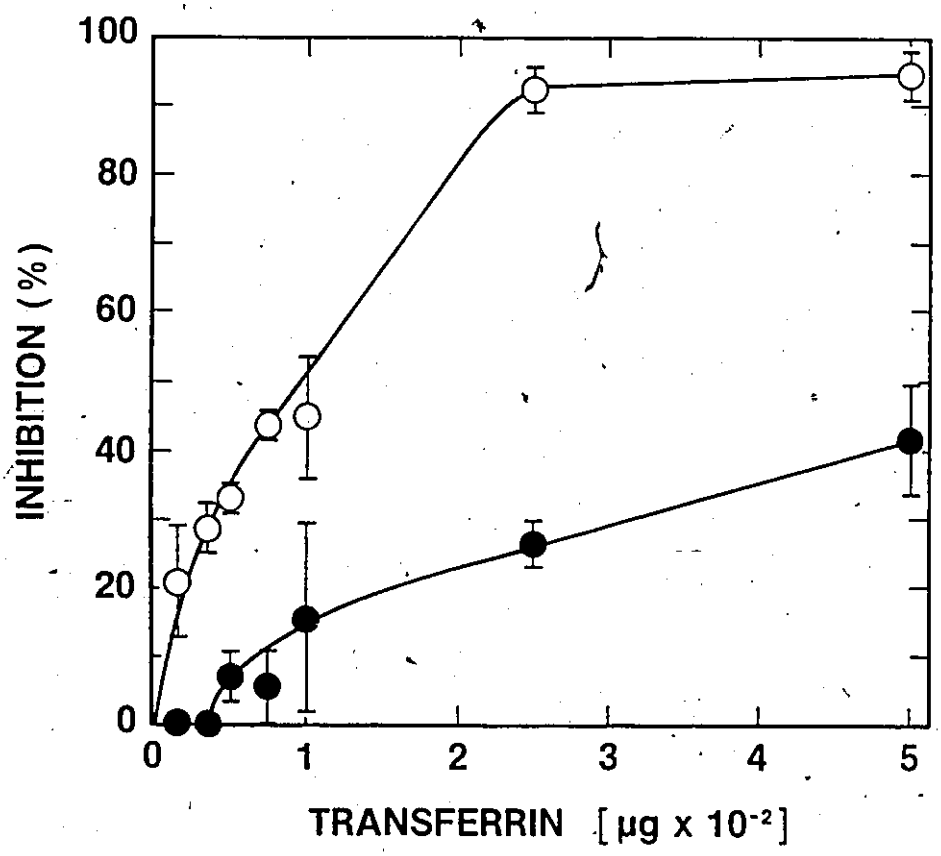


FIGURE 5.4

FIGURE 5.4 Inhibition of binding of rat ^{125}I-diferric transferrin to rat reticulocytes by human diferric transferrin (open symbols) and rat diferric transferrin (closed symbols). Cryopreserved cells, equivalent to 454 ug protein, and 0.5 ug of radioligand were used for each estimate. Binding assays were performed as outlined in Experimental Procedures (section 5.2.5.3). Points, representing specific binding, are means ± SE of measurements in triplicate.

proteins demonstrated in hepatocytes, most probably is in respect of TfRs and not some other binding site(s).

When the binding data obtained with rat Tf and reticulocytes were analysed by the method of Scatchard (1949) it was revealed that 256 ± 67 (SE) ng rat transferrin/mg cell protein were bound with an apparent association constant of 7.09 ± 0.7 (SE) $x10^7$ mol^{-1} (n=5). The parallel results from two experiments with hepatocytes revealed that the number of rat Tf binding sites was approximately 2.4×10^4 (range 1.1 to 3.7×10^4). The apparent association constant was 6.9×10^7 mol^{-1}. These values are consistent with those reported (Young et al., 1983). Hepatocytes are believed to express only a fraction of their transferrin binding sites in the cold (Cole and Glass, 1983) and as demonstrated in Chapter 8, the physiological value is higher.

Unfortunately, the corresponding values for human diferric Tf could not be calculated. It was found after considerable experimentation that iodination decreased the binding of human Tf to both rat hepatocytes and reticulocytes. The basis of this conclusion rests on the observation that dilutions of radiolabelled human Tf with unlabeled ligand resulted in a disproportionate reduction in the binding of the labelled ligand (see Figure 5.5).

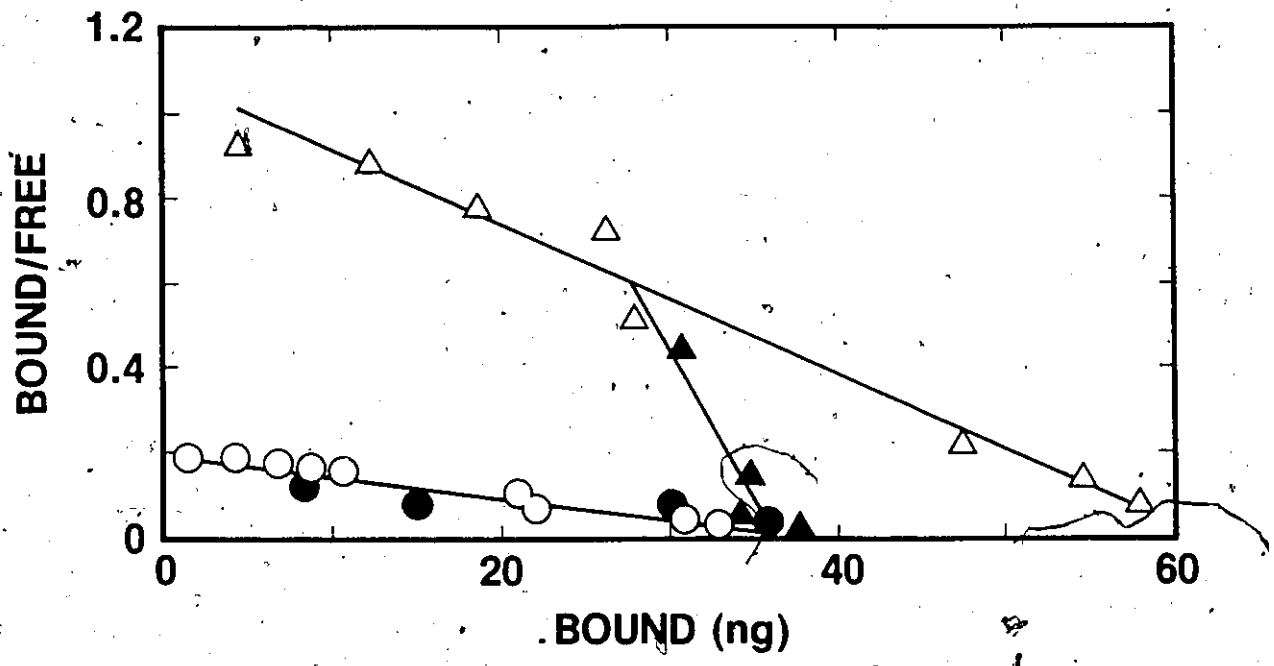


FIGURE 5.5

FIGURE 5.5 Iodination weakens the affinity of human Tf for the rat TfR. Illustrated is a Scatchard plot of the binding of radioiodinated human (Δ▲) and rat (O●) diferric Tf. Rat reticulocytes were incubated with radioligands only (open symbols) or with the radioligand diluted in the appropriate unlabelled Tf (closed symbols). The details of sampling and the assay are in Experimental Procedures (section 5.2.5.3). The values obtained with radioiodinated rat Tf were the same regardless of the presence of unlabelled material or not. On the other hand, radioiodinated human Tf bound significantly less in the presence of the homologous unlabelled protein.

Experiments were conducted with human Tf radiolabelled by an alternative procedure which does not modify the integral tyrosine residues. The Bolton-Hunter technique results in an iodinated Tf by virtue of conjugation with radioiodinated 3-(4-hydroxyphenyl)-propionic acid. However, the result was the same. These findings in sum, suggest that the reaction of human Tf with rat TfRs is affected by the presence of substituted phenolic group(s). Possibly this is because of their lowered pK values.

5.4 Discussion

5.4.1 Proposition of a Dual Receptor Uptake Mechanism based on Studies with Diferric Tfs and Asialomucin

Two significant findings from the above experiments are as follows: 1) Increasing the endogenous concentration of Tf by approximately 300% had no measurable effect on the rate of hepatic iron uptake from RAsTf; 2) The enhanced rate of iron deposition in the liver from RAsTf was completely abolished by an excess of human diferric Tf or asialomucin or both. However, the ability of RAsTf to donate iron was not abolished in an absolute sense, but was reduced to a rate comparable

to that measured for rat Tf. On the basis of these findings, it is possible to postulate a model capable of explaining the mechanism of RAsTf's preferential iron donation *in vivo*. In view of RAsTf's potential dual binding specificities it is likely that the desialylated protein gains entry preferentially to the hepatocyte interior by means of dual-receptor engagement. In particular by synergistic interaction between the liver TfR and lectin. The blockade by asialomucin clearly suggests a role for the lectin in this mechanism just as a similar blockade achieved by human diferric Tf implicates the TfR. That this mechanism cannot be just an additive mechanism as suggested by Young et al. (1983) is borne out by the fact that either singly or in combination the effect of the competitors is the same; If an additive mechanism had been operating, uptake would have been reduced by a certain portion by either asialomucin or human Tf, and the combination would have resulted in a reduction in iron uptake greater than either competitor alone could achieve. The data do not support this view.

In this synergistic interaction RAsTf most probably interacts with the lectin before the TfR. The reason for this suggestion is two fold. First, inspite of increasing the competing level of endogenous

diferric Tf there was no effect on iron delivery by RAsTf. Secondly, the combination of few, if any circulating competing asialoligands (Marshall et al., 1974) and, a much larger number of lectin binding sites (Weigel and Oka, 1983) as compared to TfR sites (Young and Aisen, 1980) on hepatocytes would argue in favour of RAsTf finding a lectin before a TfR. Secondary binding to the TfR would have to occur very soon after this initial interaction because of RAsTf's weak affinity for the lectin (RAsTf is not adsorbed by Gal/GalNAc lectin immobilized on Sepharose; see Regoeczi, 1987a). The attachment to the TfR would be responsible for the main holding effect on the ligand. How this mechanism might operate if viewed on the molecular level would be complete speculation; however, a graphic representation of the essential ideas just discussed is presented for consideration in Figure 5.6.

5.4.2 Iron Uptake by the Liver in spite of High Concentrations of Diferric Tf

The fact that iron uptake continues even in the presence of such seemingly overwhelming odds as completely saturated endogenous rat Tf; or 10 mg/100 g body weight of diferric human Tf or 23 mg/100 g body weight of diferric human Tf (see Figure 5.1 and 5.2) tends to cast

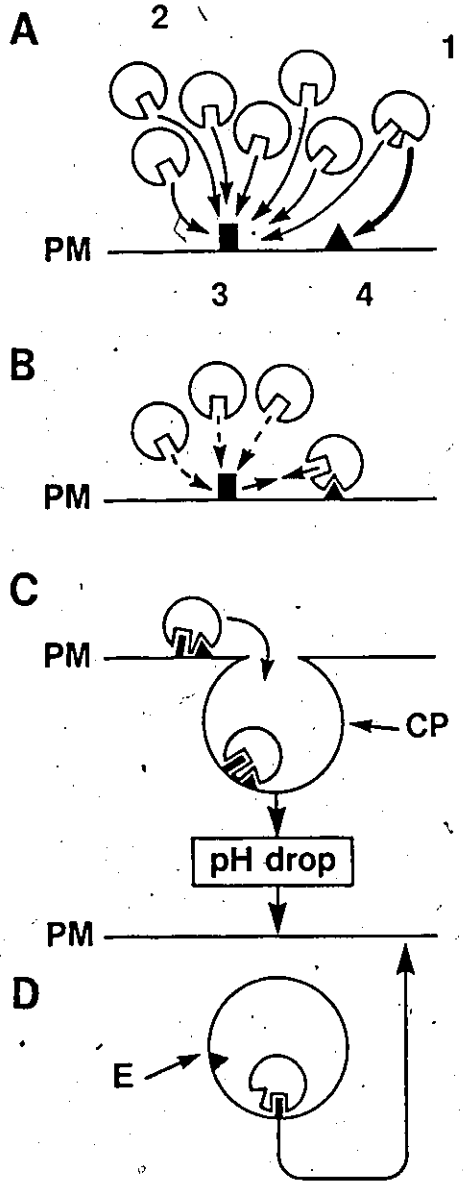


FIGURE 5.6

FIGURE 5.6 Scheme illustrating likely mechanism of diacytic cycle for rat diferric asialotransferrin. A: ligand (1) competes against a large excess of rat diferric transferrin (2) for transferrin receptor (3) at outer aspect of plasma membrane (PM). Because of its additional affinity site, rat asialotransferrin can "jump the queue" by latching on to a galactose and N-acetyl-D-galactosamine-specific lectin (4), which is more abundant and less occupied. B: lectin-bound rat asialotransferrin by virtue of its vicinity to membrane, competes for a transferrin receptor from a vantage point. C: ligand becomes ultimately bound to both receptors, and this is how it probably enters coated pit (CP). D: following endosome (E) acidification, rat asialotransferrin dissociates from lectin and follows normal subcellular route of rat transferrin.

a long shadow on the proposition that Tf mediated iron uptake by the hepatocyte proceeds only by the TfR. Indeed, it seems more likely that a large capacity bulk mechanism such as fluid-phase pinocytosis is the mode of uptake. The feasibility of such a mechanism is explored more fully in the discussion of Chapter 8 and is experimentally expanded in Chapter 9.

5.4.3 Affinity of Human Tf for Rat TfR

The finding here, that human Tf has a seven-fold higher affinity for the rat TfR was recently confirmed by Bomford and Young (1987). The implication of this finding is that subtle but significant differences in the structure of Tf can have a profound effect on the binding affinity of the Tf molecule. This was emphasised by the effects of radioiodination on human Tf (Figure 9.5). Thorstensen and Romslo (1984), have also studied the binding of human and rat Tf by adult rat hepatocytes, but the data they presented suggest that, in fact, rat Tf is the protein with a somewhat higher affinity for the TfR when compared to the affinity of human Tf. It is possible however, that their finding was due to the fact that they labelled human Tf with the Bolton-Hunter reagent. As indicated above this decreases the affinity of human Tf for the TfR (see 5.3.3).

In conclusion, the present study serves to underline the view put forward in Chapter 3 which is: Small changes in the composition of the glycan of a plasma glycoprotein may have profound effects on its physiological function. The proposition put forward here of a dual receptor mechanism is but one expression of this view. Whether the mechanism outlined above is sufficient to completely explain the observation of enhanced iron delivery *in vivo* by RAsTf, is explored further with respect to RAsTf's interactions with isolated hepatocytes in the next chapter.

CHAPTER 6

THE INTERACTION OF RAT CONTROL AND ASIALOTRANSFERRIN WITH ADULT RAT HEPATOCYTES: SUPPORT FOR THE DUAL RECEPTOR HYPOTHESIS

6.1 Introduction

Desialylation of transferrin results in its ability to preferentially donate iron to the liver (Young et al., 1983; Van Berkel et al., 1987 and see Chapters 3 and 5) and has been implicated as a possible mechanism in alcoholic hepatic siderosis (Regoeczi et al., 1984). The investigation into the mode of iron deposition to the liver by rat asialotransferrin (RAsTF) *in vivo* (as described in Chapter 5) resulted in the postulation of a synergistic dual receptor mechanism involving both the transferrin receptor (TfR) and galactose/ N-acetyl-D-galactosamine (Gal/GalNAc) specific lectin. However, work performed on isolated hepatocytes, *in vitro*, by Young et al. (1983) is at variance with this hypothesis and suggests that either the Gal/GalNAc specific lectin *or* the TfR is responsible for the uptake of RAsTF i.e. net uptake is the sum of the separate pathways. As well the work *in vitro* of Dekker et al. (1985), also differs in that it suggests that the protein can be uptaken by either receptor but that iron uptake from RAsTF can proceed only via the transferrin receptor.

Concerning the fate of RAsTf, our results *in vivo* suggested that over a 110 h period, the catabolic half-life was approximately 34% shorter than that of RTf. Observations by Young et al. (1983) in hepatocytes, on the other hand, indicated that the endocytosed desialylated transferrin was largely degraded over a sixty-minute period, particularly when the TfR was blocked. Yet another experiment by Dekker et al. (1985) showed that RAsTf was handled much the same way as RTf by hepatocytes in suspension, but that foreign desialylated transferrin, such as human asialotransferrin, was rapidly degraded, purportedly because of a foreign asialotransferrin recognition system.

The purpose of these experiments was to attempt to clarify the situation with respect to the uptake, iron donating properties and catabolism of rat asialotransferrin by hepatocytes and to further investigate the hypothesis of dual receptor-mediated uptake in the isolated adult rat hepatocyte.

6.2 Experimental Procedures

6.2.1 Materials

All materials were obtained as outlined in Chapters 2 and 3.

6.2.2 Proteins

Rat and human transferrins were isolated from the plasma of their respective sources as decribed in Chapter 2, section 2.2.3. Rhesus transferrin was prepared as described by Regoeczi et al. (1975b). The type of transferrins used with respect to their glycan content was as follows: Rat transferrin type 3, (rat transferrin type 3 "plus" (RTf-3+) was composed of ~ 80% RTf-3, ~ 19% RTf-1 and ~ 1% RTf- 2 and used to emulate conditions *in vivo*), human transferrin contained two biantennary glycans and non-fractionated rhesus transferrin. Desialylation of rat and rhesus transferrin was performed as described in Chapter 3. The desialylation of fetuin (Regoeczi et al., 1982) and submaxillary mucin was by a method described in Chapter 5.

Biologically screened rhesus AsTf and RAsTf-3 were prepared as follows. First, they were differentially labelled with ^{125}I and ^{131}I by the method outlined in Chapter 2 and each was injected i.v. into individual rats. After 20 min the animals were exsanguinated under anaesthesia. The resulting plasma was adjusted to 40% saturation with $(NH_4)_2SO_4$, stirred at room temperature for 40 min then centrifuged for 20 min at 12,060 g. The supernatant was dialysed overnight at 4°C against 2 x 1000 ml 0.1M NaI, 0.005M Hepes, pH 7.4

then a further 18 h at 4°C against 4 x 500 ml phosphate-buffered saline.

6.2.3 Isolation of Hepatocytes

Parenchymal cells were isolated from the livers of rats (200-300 g) by collagenase digestion as described in Chapter 3 and Appendix CH-3. Viability, as determined by trypan blue exclusion, was greater than 90% and contamination with non-parenchymal cells was always less than 2%.

6.2.4 Uptake Studies with Hepatocytes

Cells (range: 8-12 x 10^6/ml) contained in 20 ml glass scintillation vials were warmed at 37°C for ten min in Hank's Balanced Salt Solution (HBSS) with 1% BSA (w/v), pH 7.4 (30 min experiments) or MEM with 1% BSA (w/v), pH 7.4 (90 min experiments). Experiments were begun by addition of "Dose" solutions containing the relevant combinations of radiolabelled and competitive ligands in the particular incubation medium (see the Figure captions for details). Quantities of radio-activities added were calculated from triplicate (20 ul each) assays of the dose solutions. The cells were maintained in suspension at 37°C in a shaking water bath at 100 strokes/min. Samples of 200 ul were taken,

in duplicate, at 0, 5, 10, 20 and 30 minutes or 0, 15, 30, 60, 80 and 90 minutes, and layered on top of 200 ul of dibutyl phthalate contained in an Eppendorf tube. The tubes were centrifuged at 12,000 g for 10 sec. and 100 ul samples of the resulting cell-free medium on the top of the dibutyl phthalate were added to an equal volume of 20% trichloracetic acid (TCA) contained in 1.5 ml Eppendorf tubes, vortexed and allowed to stand for 18 h at 4°C. The tips of the minitubes which contained the cell pellets, were cut off, placed in 12x85 mm glass tubes and assayed for cell associated radioactivity. The TCA treated samples were centrifuged for 2 min and 100 ul of the resulting supernatants were transferred into 12x85 mm glass tubes for counting. All samples were assayed in a Packard model 5986 multichannel analyzer. Measurement of trypan blue exclusion at the end of each experiment demonstrated that cell viability was always greater than 85% and usually over 90%.

6.2.5 Radiolabelling

Labelling of RTf-3 and RAsTf-3 with ^{59}Fe and production of non-radioactive diferric RTf-3+ and HTf were accomplished as described in Chapter 3. Radioiodination of the proteins was carried out as in Chapter 2 except that unbound radioactivity was removed

by dialysis against 4 x 500 ml 0.1M NaI, 0.005M Hepes, pH 7.4 for 8h at 4°C followed by 4 x 500 ml phosphate buffered saline (PBS), pH 7.4, overnight at 4OC.

6.2.6 Data Treatment.

Replicate values from each experiment were averaged and fitted with curves by the computer routine of Nelder and Mead (1965). Sets of values were subjected to analysis of the variances using the IBM version of the statistical package "Minitab" (Ryan et al., 1985). Significance of the variance ratios was assessed according to the F distribution. The ANOVA tables from analysis of data corresponding to Figures 6.1, 6.2, 6.3 and 6.5 are presented in Appendix CH-6.

6.3 Results

6.3.1 Effect of Increasing Transferrin Concentration on Association with Hepatocytes

The amount of RTf-3 and RAsTf-3 found associated with hepatocytes over an increasing range of transferrin concentrations was compared (Figure 6.1). At the lowest concentration tested, (0.050 ug/ml) significantly more RAsTf-3 was cell associated than RTf-3 ($F = 7.19$; $p = 0.0179$). At each subsequent step in concentration, the

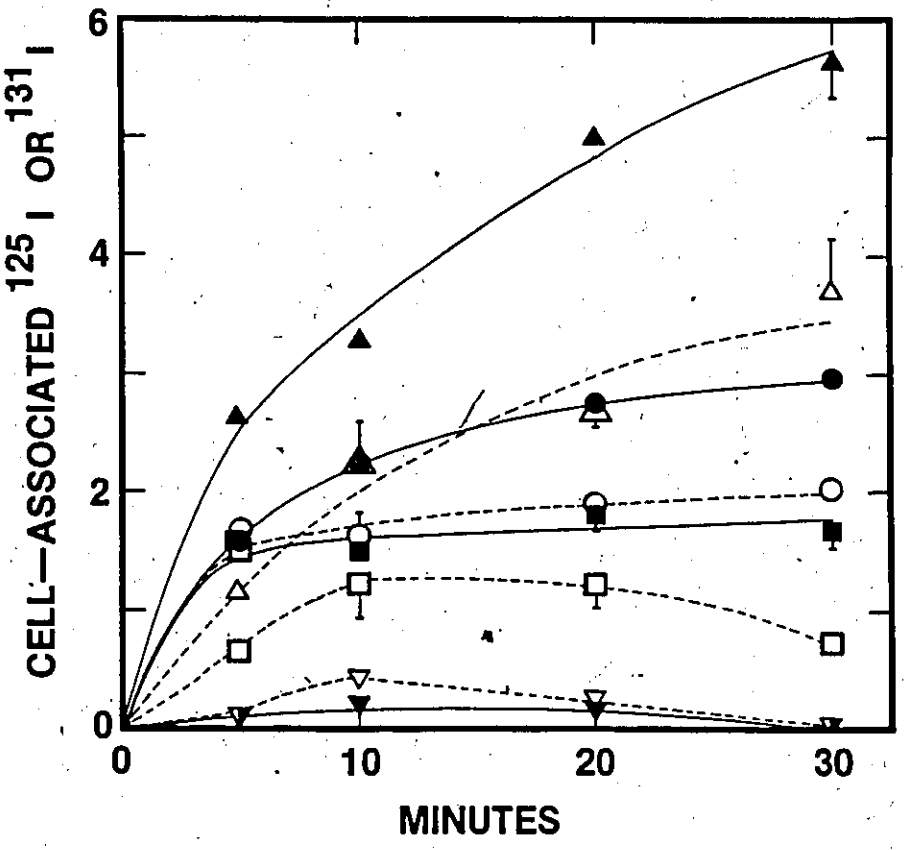


FIGURE 6.1

FIGURE 6.1 Interaction of hepatocytes with ^{125}I-RTf-3 (open symbols and discontinous curves) and ^{131}I-RAsTf-3 (closed symbols and continuous curves). Cells (9-12 x 10^6/ml) were incubated in HBSS supplemented with 1%BSA pH 7.4 at 37°C including "Doses": A) 0.05 ug/ml of each of RAsTf-3 and RTf-3 (▲ △), B) 1.0 ug/ml of each of RAsTF-3 and RTf-3 (● ○), C) 21.0 ug/ml of each of RAsTf-3 and RTf-3 (■ □) and D) 21.0 ug/ml of each of RAsTf-3 and RTf-3 (▼ ▽) in the presence of 1.55 mg/ml diferric RTf-3 (For doses in "C" and "D" each Tf type was incubated in a separate cell suspension). Samples were taken as described in Experimental Procedures (section 6.2.4). Points represent means ± SE of 2-6 estimates. Error bars fall within symbols unless otherwise indicated.

degree of difference was decreased and the percentage of the dose associated per 10^6 cells was also reduced. Experiments at the highest level, 1.55 mg/ml, which corresponded to the average concentration of diferric transferrin normally found in the plasma (Chapter 5) yielded values which were not significantly different.

6.3.2 Protein Uptake and Iron Donation in the Presence of Competing Ligands

Selective blockade of the TfR and the Gal/GalNAc receptor was performed in order to investigate their relative role(s) in the uptake of RTf-3 and RAsTf-3. Bovine submaxillary asialomucin was used to block the lectin and because of its 7-10 fold higher affinity for the rat TfR (Chapter 5; Vogel et al., 1987), diferric human Tf was used to block the TfR.

As found in the data presented in Figure 6.1, the cell-association of RAsTf-3 at a concentration of 1 ug/ml was significantly higher ($p < 0.05$) than that of RTf-3 (Figure 6.2A). Addition of 1.0 mg/ml 2[Fe]HTf caused a significant reduction in the percentage of the dose associated per 10^6 cells and virtually abolished ($F = 1.03$; $p = 0.327$) the difference in rates of association (Figure 6.2B). A similar elimination of the difference in cell-association was observed in the presence of 0.63 mg/ml of asialomucin ($F = 0.14$; $p = 0.713$), however there

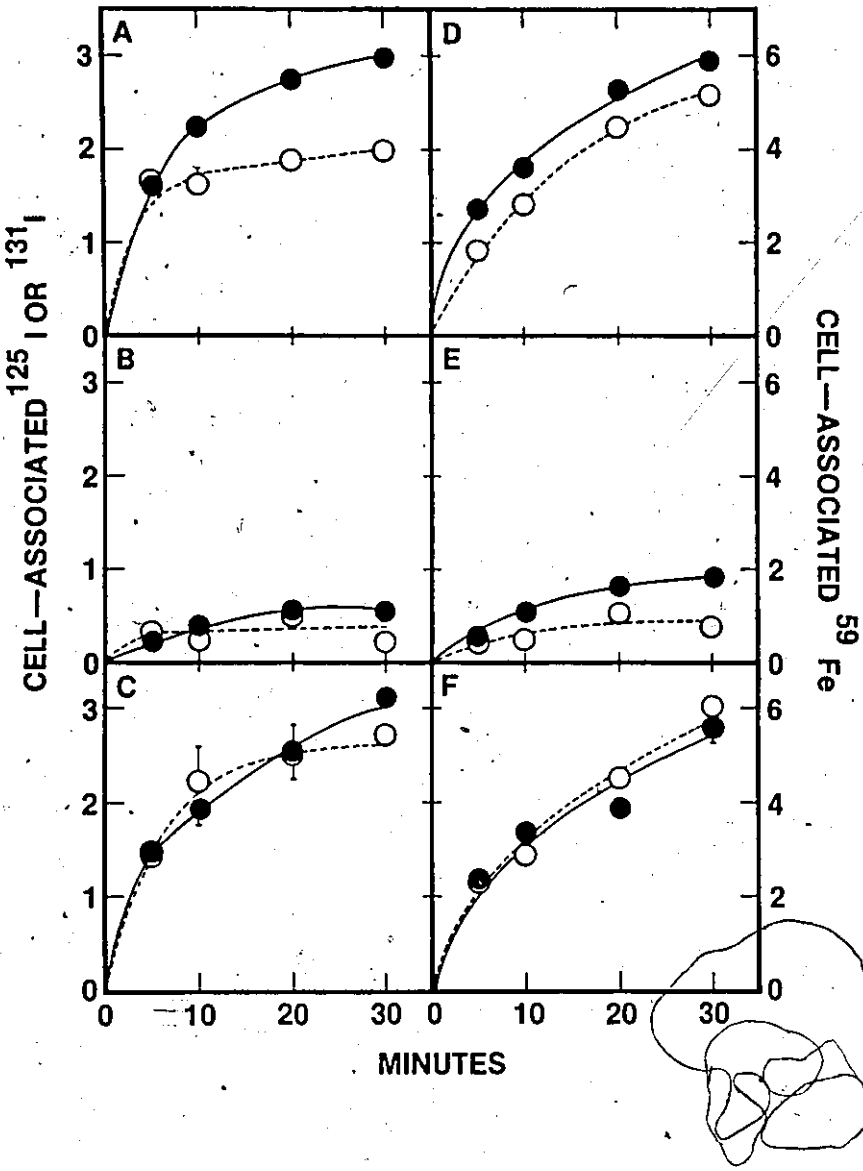

A
B
C
D
E
F
CELL—ASSOCIATED ^{125}I OR ^{131}I
CELL—ASSOCIATED ^{59}Fe
MINUTES
0
10
20
30
1
2
3
4
6

FIGURE 6.2

FIGURE 6.2 Rat Tf and rat AsTf uptake and iron donation in hepatocytes. Cells (9-10 x 10^6/ml) were incubated and samples taken as described in Experimental Procedures (section 6.2.4). "Doses" consisted of: A) 1 ug/ml of each of ^{131}I-RAsTf-3 (closed symbols) and ^{125}I-RTf-3 (open symbols) (2 ug/ml total transferrin), B) as in "A" plus 1 mg/ml diferric HTf, C) as in "A" plus 1 mg/ml asialomucin, D) 20 ug/ml of diferric (^{59}Fe)RAsTf and 20 ug/ml of diferric (^{59}Fe)RTf in separate vials, E) as in "D" plus 7.4 mg/ml diferric HTf, and F) as in "D" plus 0.64 mg/ml asialomucin. Points are mean $\pm$ SE of 2 estimates. Values are expressed as the percentage of the "Dose" per 10^6 cells. Error bars fall within symbols unless otherwise indicated.

was no reduction in the percentage of the dose uptaken compared to control (Figure 6.2C). In control experiments, to test the integrity of the lectin pathway, the uptake of asialofetuin (2 ug/ml) by hepatocytes was monitored in the absence and presence of competing loads of asialomucin as shown in Figures 6.2C and F: the competing asialomucin was found to completely block the uptake and catabolic breakdown of the asialofetuin (not shown).

The measurement of iron uptake under identical competitor conditions but at a transferrin protein concentration of 20 ug/ml (Figure 6.2D, E and F) gave qualitatively similar results except that iron donation in the presence of diferric HTf was found to be significantly different, although to a reduced extent, in RAsTf-3's favour, by 30 minutes ($F = 4.8$; $p = 0.045$). The corresponding statistics for the experiment with asialomucin were $F = 0.06$ and $p = 0.810$. Experiments conducted at the same concentrations as indicated in panels D, E and F (20 ug/ml) in which the uptake of protein was followed, gave qualitatively comparable results as seen in panels A, B and C; however the difference in uptake between proteins was not as pronounced as illustrated in Figure 6.2A (results not shown).

6.3.3 Catabolism of RTf-3 and RAsTf-3 by Hepatocytes

The accumulation of free radioiodide as TCA soluble radioactivity in the supernatant of the hepatocyte suspensions is known to reflect the catabolism of radiolabelled proteins (Regoeczi, 1987). My observations of this phenomenon in the presence of a close-to-physiological concentration of diferric RTf-3+ and then with 7.4 mg/ml diferric HTf or 0.62 mg/ml asialomucin superimposed, are summarized in Figures 6.3A, B and C. Under all three conditions, only approximately 1% of the dose per 10^6 cells appeared in the supernatant as TCA-soluble radioactivity after 90 minutes of incubation at 37°C. There were no significant differences found between the two proteins or between the different incubation conditions. Measurement of the same parameter from radioiodinated asialofetuin, in the same preparations of hepatocytes, revealed that close to 100% of the dose per 10^6 cells was available in the supernatant after 90 minutes but less than 5% was available in the presence of 0.62 mg/ml asialomucin (not shown). The measurement of TCA soluble radioactivity from radioiodinated RTf-3 and RAsTf-3, when incubated in the absence of other transferrin or asialomucin over a thirty minute period, also revealed no significant differences between proteins (not shown).

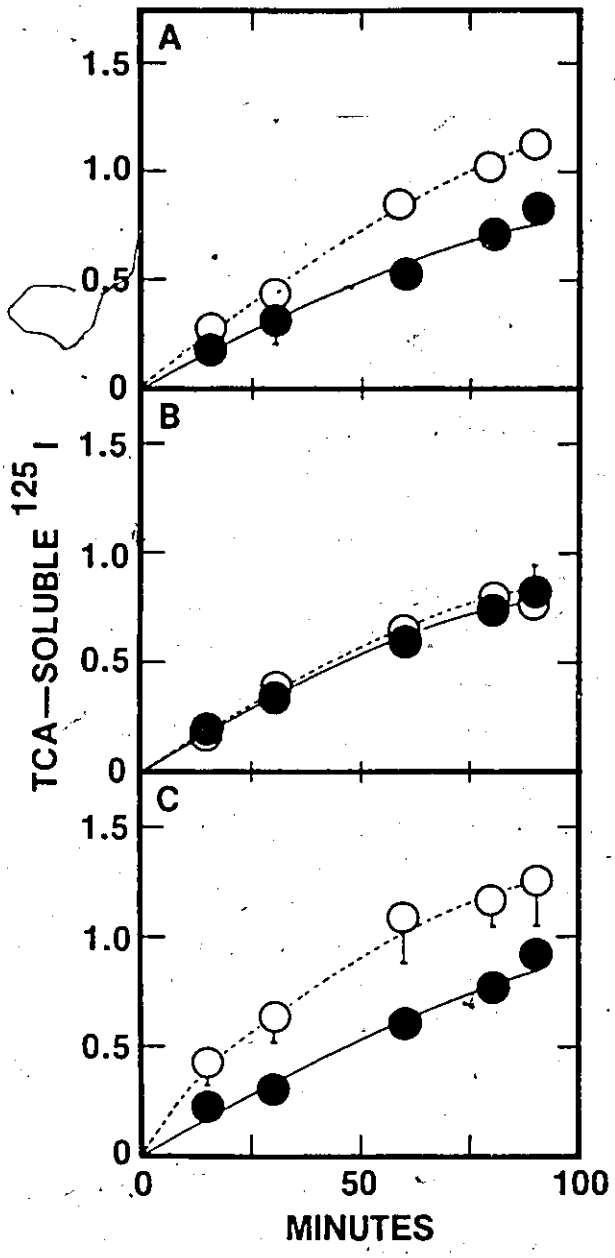


FIGURE 6.3

FIGURE 6.3 TCA-soluble radioactivity from hepatocytes incubated with rat asialotransferrin and transferrin. Cells (10-12 x 10^6/ml) were incubated in MEM containing 1% BSA pH 7.4 and 1.55 mg/ml diferric RTf-3+. Samples were taken as described in Experimental Procedures (section 6.2.4). "Doses" were as follows: A) 3 ug/ml of RAsTf-3(●) and 3 ug/ml of RTf-3(○) each in separate vials, B) as in "A" plus 7.4 mg/ml diferric HTf, and C) as in "A" plus 0.62 mg/ml asialomucin. Points are means ± SE of 4-6 estimates. Values are expressed as percentages of the "Dose" per 10^6 cells. Error bars fall within symbols unless otherwise indicated.

6.3.4 Uptake and Catabolism of RAsTf-3 and Rhesus AsTf by Hepatocytes

Rhesus transferrin was chosen to investigate the possibility of a foreign asialotransferrin recognition system in hepatocytes. This choice was based on previous work (Regoeczi et al., 1984) in which rhesus sialylated transferrin had been shown to exhibit similar liver iron donating properties as rat sialotransferrin and because of the similarity of the carbohydrate composition of the two proteins (Regoeczi et al., 1975b). Complete desialylation of rhesus transferrin required a longer period of incubation than that for rat transferrin. A preliminary study with rhesus AsTf revealed the presence of a small fraction of rapidly catabolized (denatured) molecules as illustrated in Figure 6.4. Thus to ensure the integrity of the radioiodinated preparation before use, it was submitted to biological screening (see Experimental Procedures), as was the ^{125}I-RAsTf-3.

Both proteins were found to be cell-associated to a similar extent ($F = 0.165$; $p = 0.6897$) when incubated in the absence of any other transferrin during a ninety-minute period (Figure 6.5A). Uptake in the presence of 1.55 mg/ml diferric RTf-3+ was also not significantly different between proteins over the same period, however there was a reduction in the overall percentage of the dose associated per 10^8 cells.

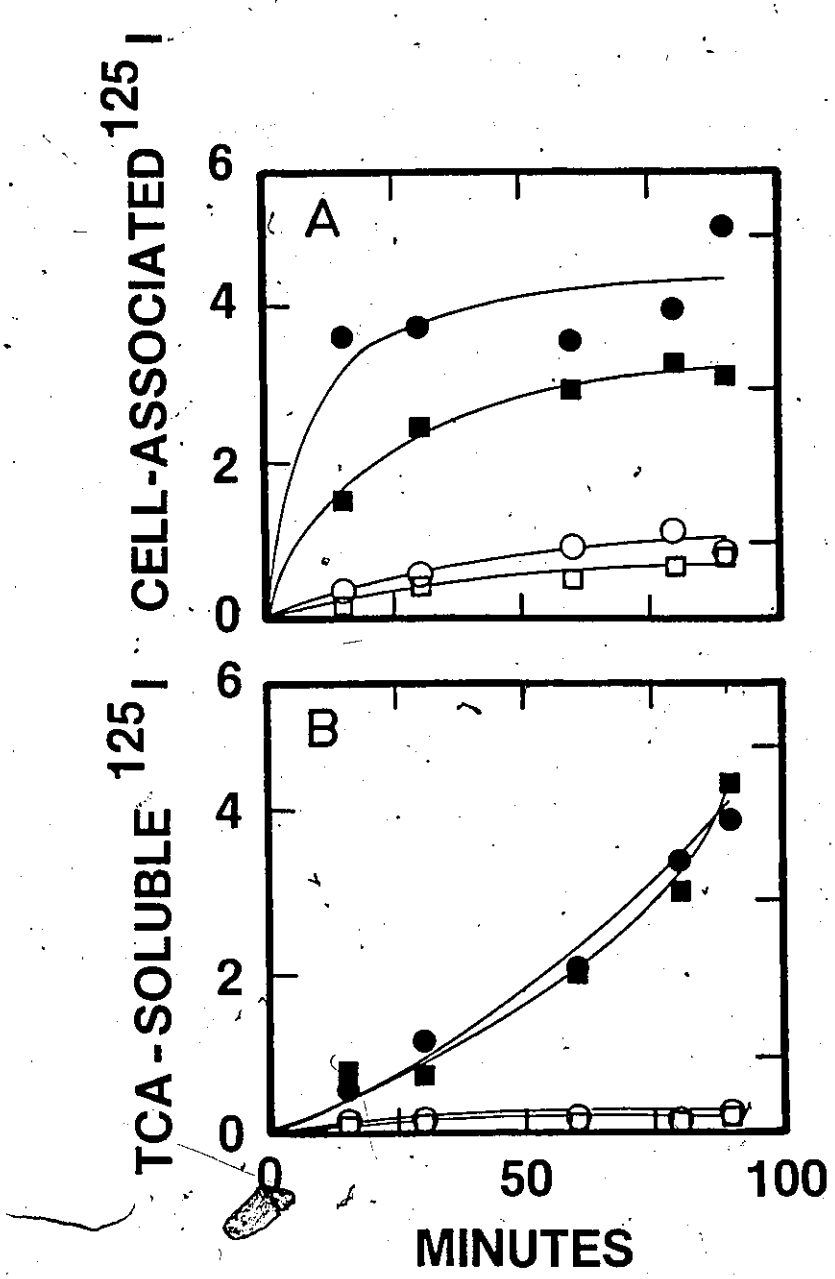

CELL-ASSOCIATED ^{125}I
TCA-SOLUBLE ^{125}I
A
B
6
4
2
0
0
50
100
MINUTES

FIGURE 6.4

FIGURE 6.4 Effect of biological screening on the association and degradation of rhesus AsTf with hepatocytes. Suspended hepatocytes (11-12 x 10^6/ml) were incubated with rhesus AsTf (0.6 - 1.6 ug/ml) either in the absence (●○) or the presence (■□) of 1.55 mg/ml diferric RTf and 1% BSA. (●■) symbols represent rhesus AsTf before screening and (○□) represent after. Upper panel (A) depicts percentage of the "Dose" associated per 10^6 cells and lower panel (B) reflects degradation expressed in the same way.

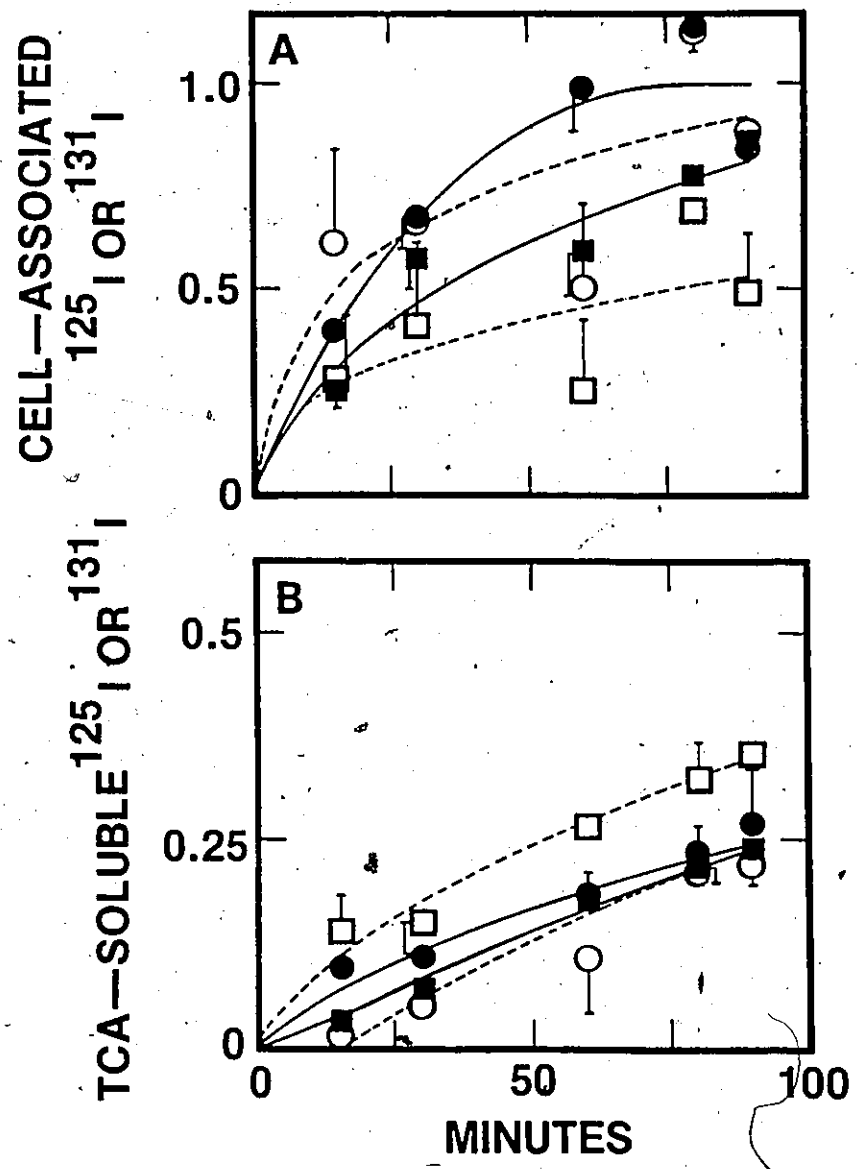

A
B
CELL—ASSOCIATED ^{125}I OR ^{131}I
TCA—SOLUBLE ^{125}I OR ^{131}I
1.0
0.5
0
0.5
0.25
0
0
50
100
MINUTES

FIGURE 6.5

FIGURE 6.5 Protein uptake and TCA-soluble radioactivity from hepatocytes incubated with biologically screened rat and rhesus asialotransferrins. Cells (11-12 x 10^6/ml) were incubated in MEM containing 1% BSA, pH 7.4 and samples were taken as described in Experimental Procedures (section 6.2.4). A) 1 ug/ml of RAsTf-3(○---○) and Rhesus AsTf(●——●) in separate vials and 2 ug/ml of RAsTf-3(□---□) and Rhesus AsTf(■——■) in separate vials each containing 1.55 mg/ml diferric RTf-3+ B) TCA soluble radioactivity from conditions described in "A". Values are means ± SE of measurements in duplicate. Error bars fall within symbols unless otherwise indicated.

The release of TCA-soluble radioiodide, from rhesus AsTf and RAsTf-3, by the hepatocytes both in the absence and presence of the near physiological concentration of diferric RTf-3+, is illustrated in Figure 6.5B. There were no significant differences found between proteins or incubation conditions during the ninety minutes of observation ($F = 1.151$; $p = 0.298$). During the same period and in the same preparations of hepatocytes, asialofetuin-derived TCA-soluble radioiodide was found to be greater than 90% of the dose per 10^6 cells (not shown).

6.4 Discussion

6.4.1 Dual Receptor Recognition

Four results from this study provide further support for the proposition that the enhanced uptake of RAsTf is mediated by a dual-receptor mechanism involving the Tf and asialoglycoprotein receptors. The first relates to evidence in support of a role for the asialoglycoprotein receptor. It is provided by data illustrated in Figures 6.2A and C where it can be seen that the preferential association of RAsTf with hepatocytes (Figure 6.2 A) was eliminated by asialomucin (Figure 6.2 C). This finding is in agreement with

experiments performed by Young et al. (1983) who also worked with isolated hepatocytes. They demonstrated that a large concentration of asialo-orosomucoid was able to reduce the number of RAsTf binding sites to numbers of sites comparable to those found for RTf.

The ability of asialomucin to reduce the enhanced uptake of radioiron from RAsTf (compare Figure 6.2D and 6.2F) is further support for a role for the lectin. This finding in vitro was entirely analogous to that observed with asialomucin and diferric (^{59}Fe)RAsTf in vivo as reported in Chapter 5.

The second finding provides evidence in support of a role for the Tf receptor. As can be seen in Figure 6.2A and B, the cell association of RTf and RAsTf was comparably reduced by a 375-fold excess of human diferric Tf. This finding too, is supported by similar observations made by Young et al. (1983). They found that HTf was able to comparably inhibit the binding of RAsTf as well as RTf to hepatocytes. It is interesting to note that RTf is approximately equipotent as an inhibitor of RAsTf (compare Figure 6.1D and 6.2B) in vitro, but was ineffective in vivo (see Chapter 5, section 5.3.1). The explanation may be due to a higher affinity of HTf for the low affinity sites as well as the TfR.

The proposition of a dual receptor mechanism is

drawn from a combination of these two findings coupled with a third result of this study. It is that a concentration-dependent relationship exists between the degree of preferential association by RAsTf and the amount of RTf. This is illustrated in Figure 6.1. and indicates that only at low concentrations of both RTf and RAsTf is the difference in their binding relationships with hepatocytes observable. The fact that increasing concentrations of RTf were able to reduce and, ultimately, to prevent the preferential association of RAsTf suggests that alternative low affinity binding sites may play a role in the RTf/RAsTf-interaction with the hepatocyte surface. The presence of such sites has been suggested (Cole and Glass, 1983 and Page et al., 1984) and indirectly demonstrated as a low affinity binding component for RAsTf by Young et al. (1983) and as a mostly non-saturable process for RTf by Trinder et al. (1986). The desialylated glycan of RAsTf expresses a low affinity for the lectin (it has been shown to be incapable of attracting a lectin immobilized on Sepharose (Regoeczi, 1987)). The lectin also expresses a hierarchy in binding constants depending on the degree of branching of the desialylated glycan; a biantennary glycan, such as that found on RAsTf-3, expresses the lowest affinity (Bezouska et al., 1985). These two

factors suggest that RAsTf's interaction with the lectin could be reasonably easily interfered with. Therefore, it is reasonable to infer that as the concentration of RTf increases, and with it the interaction with the low affinity sites, steric hindrance would also increasingly interfere with RAsTf's interaction with the lectin. It may well be that for this reason the difference in rates of cell association between RAsTf and RTf is observed only when both TfR and lectin binding sites are unoccupied (Figure 6.1). Alternatively, RAsTf may succeed in its interaction with the lectin but, because of the large numbers of Tf molecules interacting non-specifically with the cell surface, RAsTf is unable to interact with the cell surface (which may be an important second step for effective cell surface-association and ultimately internalization).

Work demonstrating the effects of both human diferric Tf and asialofetuin on the binding of RAsTf to hepatocytes has previously been reported by Dekker et al. (1985) however these investigators did not measure binding of <u>both</u> RTf and RAsTf at the same time and therefore were unable to arrive at a similar conclusion.

The fourth finding is that RAsTf is not degraded more rapidly by hepatocytes than RTf: These experiments were conducted in the presence of 1.55 mg/ml

unlabelled RTf and with a high concentration of human diferric Tf or asialomucin. None of these conditions altered the rates of degradation of either radiolabelled protein (Figure 6.3). These results suggest that RAsTf probably does not only bind the lectin or the TfR (as suggested by Young et al. (1983)) but requires both receptors for enhanced association to occur.

Only one aspect of the *in vivo* findings in Chapter 5 (which allowed the proposition of a dual receptor mechanism) was not reproduced *in vitro*; i.e., an enhanced uptake of RAsTf in the presence of diferric RTf. One possible explanation for the discrepancy may be that *in vivo* measurements do not distinguish among hepatic cell types. Apart from hepatocytes, the liver is composed of endothelial cells as well as Kupffer and stellate cells (Blomhoff et al., 1984). Recently van Berkel et al. (1987) showed that both endothelial and Kupffer cells, in addition to the hepatocytes, can ingest RAsTf. Thus, the combination of cells taking up RAsTf may account for the desialylated protein's evidently higher rate of uptake *in vivo*.

A further possibility is that the dual receptor mechanism cannot operate in the absence of the histological conditions which prevail in the normal liver. In the intact liver the hepatocyte faces the space of Disse

which is a mixing pool between plasma and lymph (Smallwood et al., 1968). The space has been found to be less accessible to plasma albumin than to small molecules like sucrose and inulin (Goresky, 1981). Correspondingly, the Tf concentration in the space of Disse is likely to be lower than in the plasma.

In any event, it isn't entirely clear that the dual receptor mechanism is totally responsible for the results. The data in Figure 6.2E indicates that in spite of a 375-fold excess of human diferric Tf, iron uptake by hepatocytes from RAsTf still exceeded that for RTf. Possibly the lack of sialic acid may play a role in enhancing iron release to the cell by methods other than the TFR such as reduction of iron at the cell's surface as suggested by Morley and Bezkorovainy (1985b) and Cole and Glass (1983). Certainly a buffer-mediated pH decrease does not lead to a more rapid release of iron from asialo Tf versus Tf (Chapter 4) however examination in vitro of these alternative sytems may reveal differences between RAsTf and RTf.

6.4.2 Receptor for Heterologous Asialotransferrins

If a receptor for foreign asialotransferrins were present on rat hepatocytes, as proposed by Dekker et al. (1985), then the uptake and catabolic rates of rhesus

AsTf should have exceeded those of RAsTf-3. As illustrated in Figure 6.5, this clearly was not the case. In fact, the catabolism of rhesus AsTf was found to be very similar to that of RAsTf-3 and this is probably because their respective glycans have comparable affinities for the lectin. The phenomenon observed by Dekker et al.(1985) - rapid uptake and catabolism of human AsTf versus RAsTf - is better explained by the ability of different glycan structures to give rise to asialotransferrin's different affinities for the lectin (Debanne et al., 1981). As well, there are different affinities of Tf from different species for the TfR. (Penhallow et al., 1986; Chapter 5).

6.4.3 Biological Screening

The increasing trend in scientific investigation towards the use of protein-cell incubation systems provides the background for presentation of the findings with biologically screened radiolabelled ligands. The integrity of these radiolabelled proteins is of concern, because, as can be seen in Figure 6.4 the presence of even a small fraction of denatured material in the labelled preparation can give rise to completely erroneous results in short term <u>in vitro</u> studies. This is to be contrasted with studies <u>in vivo</u> where such

material is rapidly removed from the circulation. As well, in long term studies the degradation of a larger portion of non-denatured material is measurable and thereby distinguishable from that which was denatured.

A striking result of the experiments reported above concerning transferrin uptake in the presence of excess transferrin, which further parallels the in vivo system, was that in spite of the large excesses of competing diferric Tfs (both human and rat), association of the protein and uptake of iron continued. As has been noted, (Bomford and Munro, 1985) adult hepatocytes have far fewer TfRs in comparison to maturing erythroid cells. Thus, under most of these conditions TfRs were likely saturated. This raises the following question: Just what is the role of the TfR in the adult hepatocyte with respect to Tf-mediated iron delivery? A significant portion of the remaining three chapters is devoted to exploring this question.

CHAPTER 7

ISOLATION OF THE RAT TRANSFERRIN RECEPTOR

7.1 Introduction

The uptake of RAsTf by the liver has been proposed to occur by a synergistic mechanism of action between the transferrin receptor (TfR) and the asialoglycoprotein receptor (see Chapters 5 and 6). However the impression thus far of the role of the TfR has been based on studies with human transferrin which only competitively blocks the receptor and thus the extent of the receptor's contribution to the postulated dual receptor mechanism is unclear.

A second area of uncertainty concerning the TfR stems from the belief that it is the most important mechanism by which transferrin (Tf) delivers its iron to hepatocytes (Nunes et al., 1986; Bomford and Munro, 1985; Young et al., 1983; Grohlich et al., 1977). However this belief may not necessarily be warranted in the light of the results reported in Chapters 5 and 6 and on the basis of findings by others concerning non-receptor mechanisms (Cole and Glass, 1983; Baker et al., 1985; Page et al., 1985).

A final issue to be dealt with here in respect of the TfR concerns the fact that, the unique feature of the TfR to bind diferric Tf at pH 7.4 and apo Tf at pH 4.5-6.5, but release apo Tf at pH 7.4 has only been demonstrated in reticulocytes (May and Cuatrecasas, 1985) Thus far this has as yet to be demonstrated in liver tissue.

In respect of these issues, experiments designed to: 1) characterize the pH dependence of Tf binding to liver plasma membrane, and; 2) to measure iron uptake in the presence of blockade of the TfR's binding site, would be effective means of further elucidating the TfR's mechanism in the liver (i.e., is it the same as the pH-dependent binding of Tf as demonstrated in reticulocytes?) and determining the receptor's contribution to iron uptake from Tf in the liver. Concerning the second aspect, total blockade would be more conclusive in deciding the TfR contribution to the dual receptor mechanism than experiments with human Tf which acts only as a competitive inhibitor (Chapter 5 and 6).

In the light of the above, isolation of the TfR from adult rat liver was deemed desirable with the following objectives in mind: 1) to provide further biochemical evidence for its existence in the liver and understand its mechanism of interaction with transferrin

and 2) to allow for production of an antiserum which would, potentially, contain IgG able to block the binding site.

The TfR is a glycoprotein which consists of two 93-KDa subunits, each of which is capable of binding one molecule of Tf (Newman et al., 1982). It has now been isolated from several species including chicken (Schmidt et al., 1985), man (Wada et al., 1979; Seligman et al., 1979; Hamilton et al., 1979; Loh et al., 1980; Trowbridge and Omary, 1981; Sutherland et al., 1981; Stein and Sussman, 1983; Tsundoo and Sussman, 1983) mouse (Van Driel et al., 1984) , rabbit (Leibman and Aisen, 1977; Van Bockxmeer and Morgan, 1977; Light, 1978; Ecarot-Charier, 1980), rat (Fernandez-Pol and Klos, 1980; Jeffries et al., 1985) and sheep (Pan and Johnstone, 1983; Pan et al., 1983).

The first step in TfR purification is membrane solubilization, and has been done mostly by Triton X-100, but deoxycholate (Trowbridge and Omary, 1981), Nonidet P-40 (Sutherland et al., 1981), or Teric 12A9 (Van Bockxmeer and Morgan, 1977) have also been used. Subsequently, the receptor is separated from the lysate by employing one of the following techniques: (1) simple gel filtration in the presence of labelled Tf (Leibman and Aisen, 1977; Van Bockxmeer and Morgan, 1977);(2) indirect immunoprecipitation by antibodies to Tf (Wada et

al., 1979; Hamilton et al., 1979; Enns and Sussman, 1981a and 1981b) in the presence of a saturating amount of Tf;(3) direct immunoprecipitation with antibodies to the TfR (Sutherland et al., 1981; Enns and Sussman, 1981b; Enns et al., 1981; Schneider et al., 1982; Van der Heul et al., 1982);(4) immunoaffinochromatography using immobilized antibodies to the TfR (Trowbridge and Omary, 1981; Jeffries et al., 1985) or to Tf (Seligman et al., 1979) and (5) affinity chromatography using immobilized diferric Tf (Schmidt et al., 1985; Seligman et al., 1979; Fernandez-Pol and Klos, 1980) or deoxycholate-coupled poly(L-lysyl)agarose (Larrick and Cresswell, 1979).

The disadvantages of approaches 3 and 4 above are that dissociation of the receptor-antibody complex requires either harsh conditions with respect to pH (Jeffries et al., 1985; Pan et al., 1983) or the use of a mixture of detergents (Trowbridge and Omary, 1981). The other techniques accomplish dissociation of the transferrin-receptor complex by using excess transferrin (Light, 1978) or 22% polyethylene glycol (Tsundoo and Sussman, 1983); however, subsequent removal of these agents is cumbersome (Witt and Woodworth, 1978) and prolongs the procedure.

Initial work to isolate the receptor from liver tissue was performed by methods similar to those

described by Ecarot-Charrier et al. (1980) which employed affinity chromatography with immobilized diferric transferrin. However this work was met with limited success.

In the light of the problems hampering isolation of TfR from liver, the rat reticulocyte was used as an alternative source of receptor. Compared to the techniques indicated above, the one-step procedure proposed by Van Driel et al. (1984) (see Experimental Procedures) appeared to be a major simplification because it takes into account the physiology of transferrin-TfR interactions as established by Morgan. (Outlined in detail in Chapter 1.) However, there were a number of problems which arose as a result of using this methodology. The technique which proved to be most successful and the solutions to the problems encountered during the isolation of the receptor are described below.

7.2 Experimental Procedures

7.2.1 Materials

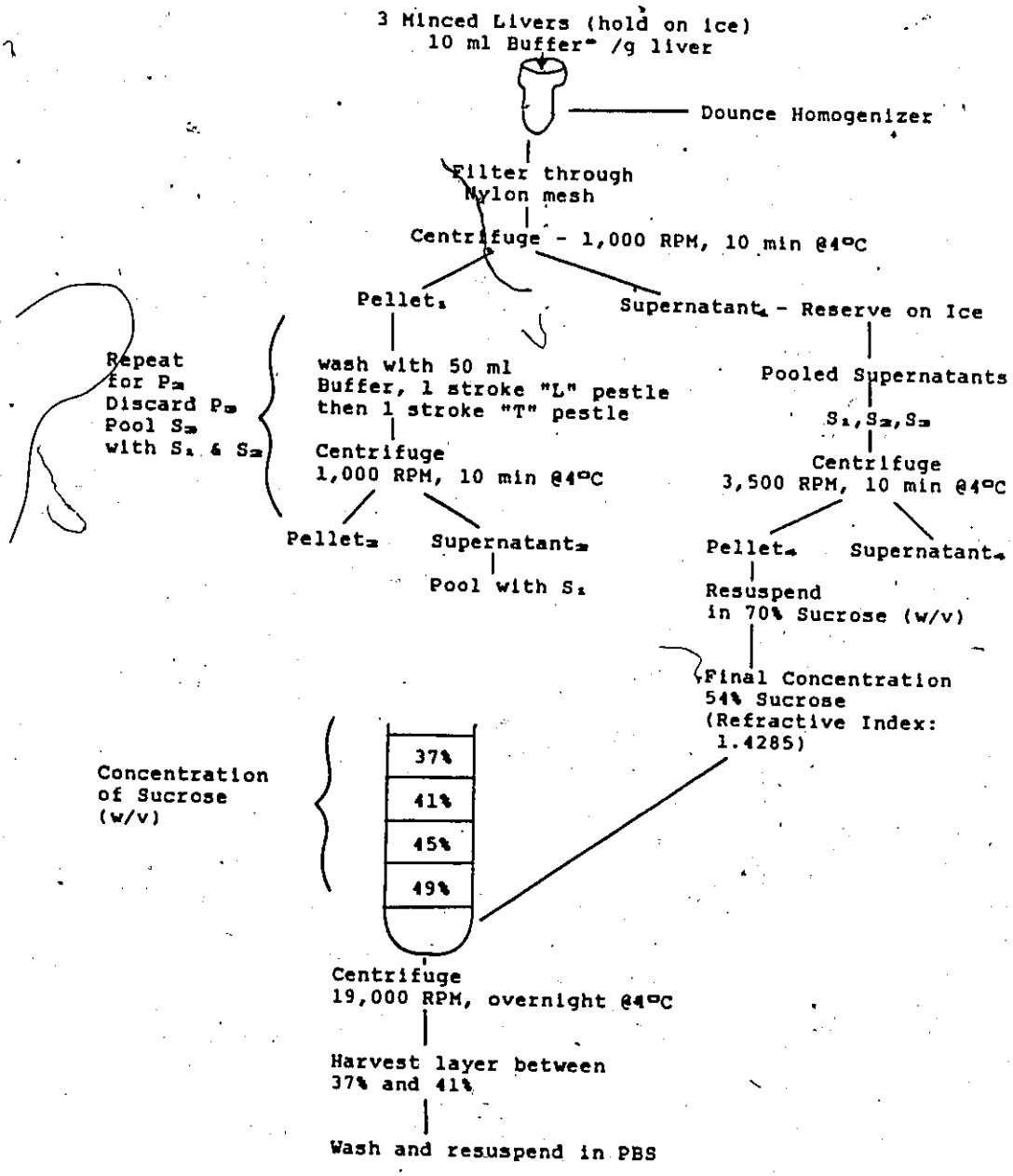
Human IgG was obtained from Connaught Laboratories (Toronto, Ont.). All other materials were obtained as outlined in Chapters 2 and 3.

7.2.2 Preparation of Plasma Membranes

The preparation of the liver plasma membranes was according to the procedure outlined by Dorling and LePage (1973) and is schematically presented on the following page. Livers (12-18 g) from three rats (150-200 g) were used for each preparation, each of which was perfused with ice cold 0.05M NaH_2PO_4, 0.5M NaCl, pH 7.4 before mincing and Dounce homogenization with six strokes of an "L" pestle. The final preparation was stored in 2 ml portions at -40°C until further use.

7.2.3 Binding Studies

Radiolabelled (see Chapter 2 for procedure) human diferric and apo transferrins were used to determine the dependence of binding on pH. For each assay, total and non-specific binding was performed in triplicate and duplicate respectively over a pH range of 4.5 to 10.0. Apo transferrin was maintained iron free by addition of Desferal to the incubation medium at a final concentration of 2.5 mM. A 500-fold excess of unlabelled human transferrin was used to determine non-specific binding. The final volume of each sample was 200 ul and the pH range was obtained with three buffer systems: 1) pH range 4.5 to 6.0 using citric acid - trisodium citrate; 2) pH range 5.5 to 8.0 with NaH_2PO_4-Na_2HPO_4 and 3) pH 8.0 to



*Buffer= 1mM $Na_2B_4O_7$, 0.5mM $CaCl_2$, pH 7.5

(after Dorling and LePage, 1973)

10.0 using a KCl - H_3BO_3 and NaOH system. In all cases 1-O-methyl B-D galactopyranoside, BSA and NaCl were included at a final concentration of 20 mM, 0.5% and 0.125 M, respectively. Experiments were conducted at room temperature and began with a 15 minute pre-incubation of the plasma membranes used to measure non-specific binding (in duplicate) with unlabelled human transferrin (apo or diferric depending upon the experiment) at a concentration 500 fold greater than the labelled material. The radioiodinated transferrin was then added to all samples (for total-in duplicate- and non-specific binding) and the incubation continued a further 30 min. Experiments were terminated by centrifugation, including a single wash with the relevant buffer (ice cold). Tips containing the pellet were severed from the Eppendorf tubes and counted in a Packard Model 5986 multichannel analyzer.

7.2.4 Solubilization of the Receptor

Washed reticulocyte ghosts were obtained as described in Chapter 3. They were solubilized in phosphate-buffered saline (PBS), containing 1% Triton X-100 and aprotinin (Sigma: 15 ug/ml), at a final concentration of 1 mg cell protein/ml. After 1 h on ice under constant stirring, the insoluble material was removed by

centrifugation (141 000 g for 1 h or 30 877 g for 2 h) at 2-4°C. The supernatant, diluted 1 in 5 with PBS, was used for affinity chromatography.

7.2.5 Affinity Chromatography

Affinity adsorbents were prepared by coupling rat or human diferric transferrin to Sepharose 4B according to the method of Porath et al., (1973). The proteins were isolated from plasma according to the methods outlined in Chapter 2 section 2.2.3. Columns, containing approximately 10 mg of ligand per gram of wet gel, were operated at 5°C essentially as described by van Driel et al. (1984). Briefly, the solubilized TfR was loaded and adsorbed at pH 7.4; then the column was rendered iron-free by washing with 500 ml of 0.1M citrate buffer, pH 5.0, containing 0.2% Triton X-100 and 50 ug/ml of the specific iron chelator, Desferal. The alkaline buffers used to elute the adsorbed TfR were: 1) 0.5M ammonium carbonate pH 7.8 (as employed by van Driel et al., 1984) and 2) 1 mM Tris-HCl, pH 8.0, containing 1M sodium iodide. (The sodium iodide was added just before elution.) The buffers each contained 0.2 % Triton X-100 and 50 ug/ml of Desferal. The efficacy of elution was monitored by measuring rat ^{125}I-labelled transferrin-binding activity in the fractions. The assay used to

assess binding activity was performed as follows: Samples (0.2 ml) were incubated for 40 min. at room temperature with the radioligand in the presence of 1% bovine serum albumin and then placed in an ice bath. Human immunoglobulin G (0.2 ml; 1.5 mg/ml of PBS) was added, followed by 0.2 ml of a saturated solution of ammonium sulphate, pH 7.4. After 15 min precipitates were separated by filtration through Whatman GF/C microglass filters and two washings (4 ml each) with 40% ammonium sulphate, pH 7.4. A 20-fold excess of human diferric transferrin was used to measure non-specific binding. Filters were counted in a Packard Model 5986 multichannel analyzer. Residual TfR that remained on the affinity columns after different elution schemes was assessed by polyacrylamide gel electrophoresis in sodium dodecyl sulphate as described by Janatova and Gobel (1984).

7.3 Results

7.3.1 pH Dependence of Tf Binding to Liver Plasma Membranes

The technique of separating bound versus free ligand used in these experiments was centrifugation. The reason was that preliminary assays demonstrated a marked increase in non-specific binding, particularly at lower

pH values, using the filtration technique (results not shown). This was possibly due to a change in ionic interaction or a decrease in the hydrophobic character of the protein, either of which, or both, may have enhanced adsorption to the filter material. This observation is reported here only as a reminder of the importance of trying a variety of separation techniques when setting up a binding assay in order to establish the method most representative of the actual binding activity.

Human transferrin was used in this study because of its seven-fold higher binding affinity for the rat TfR (see Chapter 5, section 5.3.3). As can be seen in Figure 7.1 the binding of apo and diferric transferrin was comparable over the pH range of 4.5 to 5.5, however marked differences were observed as the apo transferrin binding was found to be maximal at pH 6.5 while diferric bound only 50% of its maximum. In sharp contrast, at pH 7.0 and at pH 7.4, apo transferrin exhibited essentially no binding activity while di ferric Tf reached its maximum. Both the iron laden and iron free forms of the protein were bound to some extent at pH 9.0-9.5, diferric Tf more than apo Tf. Finally, binding decreased sharply for both diferric and apo Tf respectively at pH 10.0.

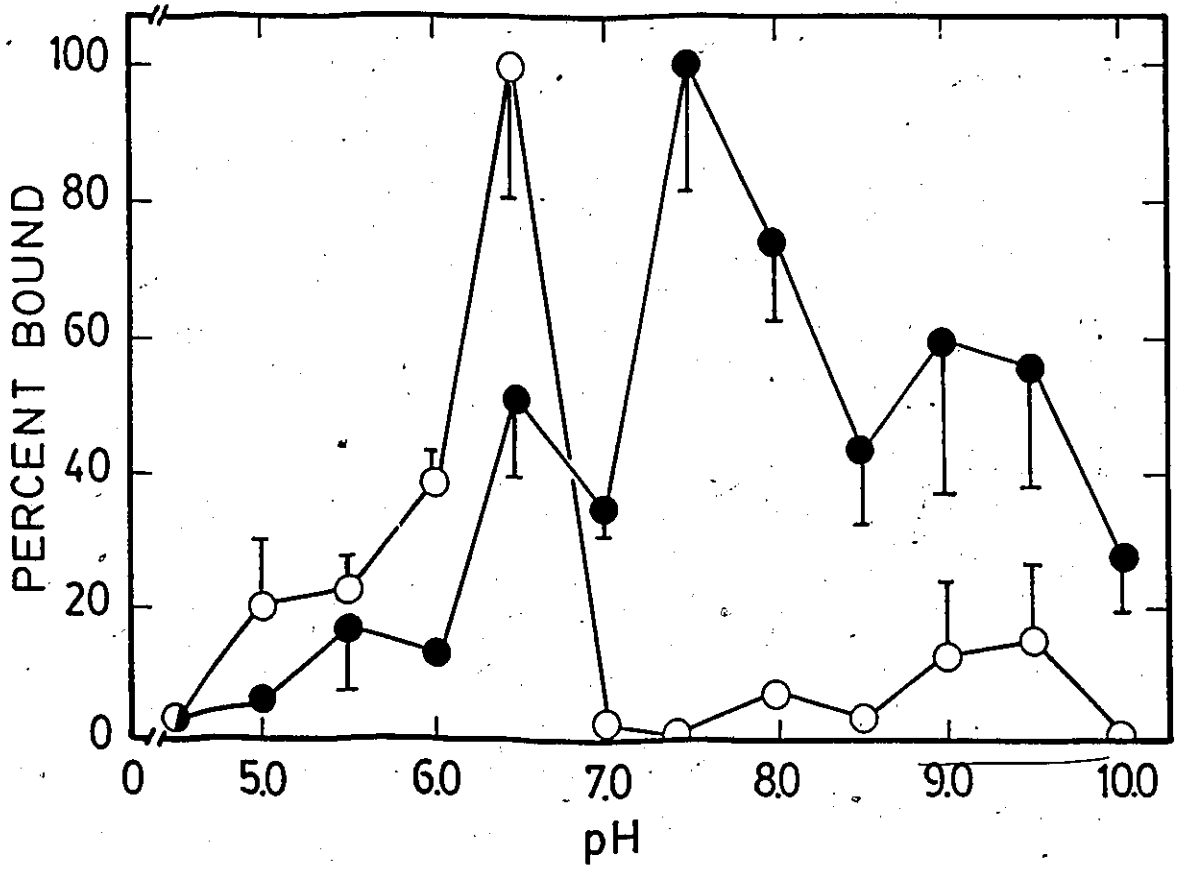


FIGURE 7.1

FIGURE 7.1 pH-dependence of Tf binding to liver plasma membranes. Either human diferric (●) or apo (○) transferrin (0.0121 uM) was incubated with 100 ug of liver plasma membranes under different conditions with respect to pH (see Experimental Procedures - section 7.2.3). Results are presented as the percentage of maximum binding obtained over the range of pH values within each experiment. Points represent the means ± SE of 3 experiments. See text for further details.

7.3.2 Isolation of the Receptor

Contrary to any expectations based on the above observations, the elution technique suggested by Van Driel using ammonium carbonate completely failed to release rat TfR from affinity columns with HTf as the adsorbent. This was confirmed by by electrophoretic examination (Janatova and Gobel, 1984) of a sample of the column material after elution (results not shown). The recovery of receptor was similarly poor from homologous affinity columns when elution was attempted on the basis of unequal affinities of TfR for apo- and holotransferrin at pH 7.4. This is evident from the dragging appearance of small quantities of receptor activity on elution with ammonium carbonate as seen in Figure 7.2. Examination of the contents of the affinity column by polyacrylamide gel electrophoresis before and after elution showed a substantial quantity of TfR still adherent to the adsorbent after attempted elution (Figure 7.3, top). On the other hand, elution of TfR from the apotransferrin column was greatly promoted by 1M sodium iodide. As seen in Figure 7.2, the receptor appeared in the chromatogram as a sharp peak. Complementary to this finding, little residual TfR remained electrophoretically detectable in the gel at the end of the run (Figure 7.3, bottom). Both rat and human Tf were capable of adsorbing all Tf binding activity from the starting material.

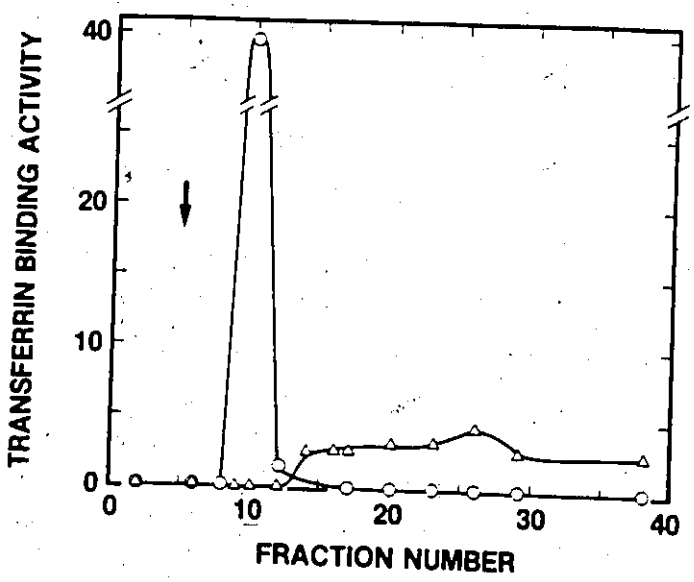

TRANSFERRIN BINDING ACTIVITY
40
20
10
0
0
10
20
30
40
FRACTION NUMBER

FIGURE 7.2

FIGURE 7.2 Elution of the rat transferrin receptor from a column (11 cm x 1 cm I.D.) of rat transferrin-Sepharose by two eluents. Before elution, the column was exhaustively washed at pH 5.0 to remove iron from transferrin as described in Experimental Procedures (section 7.2.5)(not shown). Arrow denotes the beginning of specific elution either with 0.5M ammonium carbonate, pH 7.8 (△), or 1 mM Tris-HCl, pH 8.0, containing 1M sodium iodide (○). Results are expressed as nanograms of ^{125}I-labelled transferrin bound specifically by 50-ul portions of the fractions tested.

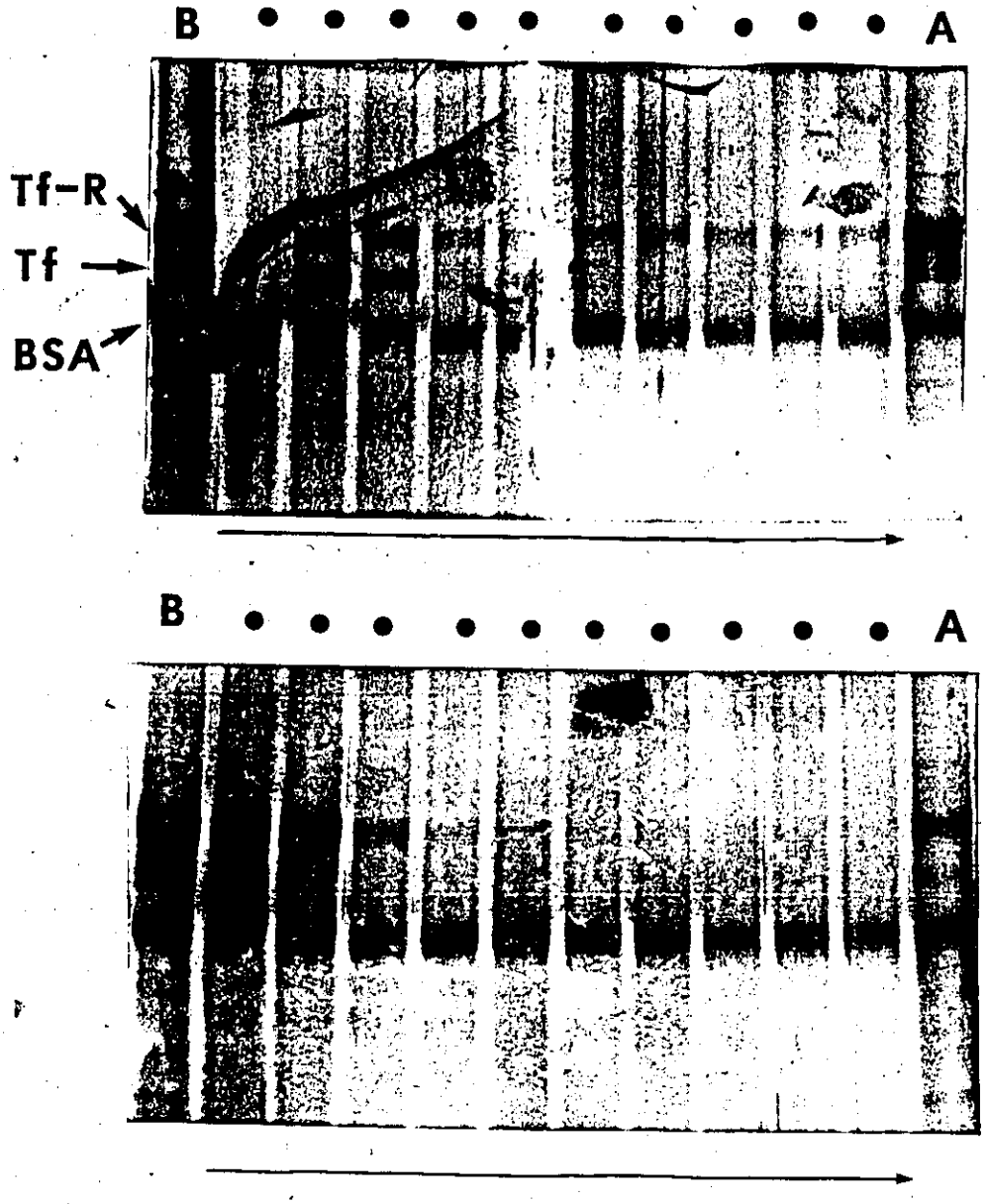


FIGURE 7.3

FIGURE 7.3 Evaluation by polyacrylamide slab gel electrophoresis of two methods of eluting the rat TfR from a column (11 cm x 1 cm I.D.) of rat transferrin-Sepharose. Elution in the top was by 0.5M ammonium carbonate, pH 7.8, and in the bottom by 1 mM Tris-HCl, pH 8.0, containing 1M sodium iodide. Migration from top to bottom. Silver staining (Heukeshoven and Dernick, 1985). Track B was loaded with a sample of the affinity gel before elution, and track A after elution. Tracks designated by dots in the upper gel were loaded with samples from fractions No. 12, 14, 16, 18, 20, 23, 26, 29, 31 and 33, and in the lower gel from fractions 9, 10, 12,14, 16, 18, 20, 22, 24 and 26 of the chromatogram in the order as indicated by the arrows. TfR, transferrin receptor; Tf, transferrin; BSA, bovine serum albumin. Note the difference between both runs with respect to the TfR content of the fractions as well as of tracks A.

7.4 Discussion

The binding data presented in Figure 7.1 show that the liver plasmalemma possesses Tf binding activity and that the character of this activity with respect to pH dependence is similar to what has already been described for the TfR in other systems, such as K562 cells (Klausner et al., 1983; Dautry-Varsat et al., 1983) and the reticulocyte (Morgan, 1983). Indeed the maximal binding activity of apo Tf at pH 6.5 and diferric Tf at pH 7.4 to adult rat liver plasma membranes compares well with these reports. Recent studies of the circular dichroism (CD) spectroscopy of diferric and apo Tf at various pH values have offered some insight into the explanation for Tf's behaviour in such conditions. Teeters et al. (1986) have demonstrated that the CD pattern of apo Tf at pH 4.5 is similar to the pattern corresponding to diferric Tf at pH 7.0. In contrast, the CD pattern of apo Tf at pH 7.0 is noticeably different. The authors suggest that this pH controlled variation in structural conformation may be responsible for Tf's ability to remain bound to its receptor at acidic pH values. It is tempting to speculate from this, that as human diferric Tf loses a single atom of iron at pH 6.5 (Lestas, 1976) it may obtain a conformation partly

similar to that of apo Tf. Thus accounting for the observed 50% of maximum binding by "diferric" Tf at pH 6.5.

Regarding isolation of the receptor, the discrepancy between these results and those of Van Driel et al. (1984) is likely explained by intrinsic differences in affinities among transferrins and TfRs of various species. In contrast to our homologous system, the above authors worked with human transferrin and murine TfR. The recognition that significant interspecies differences exist with repect to TfR-transferrin interactions, is very recent (Rudolph et al., 1986; Tsavaler et al., 1986; Penhallow et al., 1986 and Vogel et al., 1987). It is not surprising therefore that the association in a heterologous affinity-chromatographic system may turn out to be either stronger (Rudolph et al., 1986) or weaker (Tsavaler et al., 1986) than in a homologous setting. This may present an advantage, or disadvantage, depending on the combination selected. In spite of this, it is curious that the rat receptor-human ligand system has such a different degree of affinity compared to the murine-human combination given the phylogenetic relationship of the rat and mouse. Ultimately, the molecular structures of the receptors will help decide the reasons for this behaviour.

I^-, together with ClO_4^- and SCN^-, belongs to the group of chaotropic ions. These large negative anions have been used previously for the dissociation of primary antigen-antibody complexes and the unfolding of macromolecules (Dandliker et al., 1967). These dissociations are achieved largely due to the anion's capability of electrostatic shielding and reducing hydrophobic bonding. The results presented above indicated that I^-, at the relatively low concentration of 1M, is highly efficient in disrupting the bonding between rat transferrin and its receptor without causing irreversible denaturation of the receptor. Potassium thiocyanate has also been used in a similar context (Fernandez-Pol and Klos, 1980) but its use is less desirable due to the difficulty of removing the thiocyanate ion from proteins. This presents a collateral problem in that even a trace of thiocyanate causes resistance to iodination (Regoeczi, 1987).

Given that the TfR had been successfully isolated it was possible to attempt to produce antibodies with blocking activity. The antibodies produced, and their use to gain further insight into the role of the TfR in liver, forms the subject material of the next chapter.

CHAPTER 8

QUANTIFICATION OF RAT HEPATOCYTE TRANSFERRIN RECEPTORS WITH POLY- AND MONOCLONAL ANTIBODIES AND PROTEIN A

8.1 Introduction

As suggested in the last chapter, part of the objective of this work was to produce an antibody which blocked binding of Tf to the receptor. Such a tool would help to directly establish the role played by the receptor in promoting the preferential uptake of iron from RAsTf (in comparison with uptake from RTf, see Chapters 3, 5 and 6). However, preliminary work with antisera to the receptor demonstrated no blocking activity whatsoever. Therefore it was decided to utilize the immunoreagent for the quantification of hepatocyte TfRs. All previous work in this area was based on binding studies with either apo or diferric transferrin (Tf) on hepatocytes either in culture or suspension. The resultant values range from 5,000 to 63,000 surface receptors per cell, with the preponderance of the estimates in the order of 2-4 x 10^4 per cell. The uncertainty which this range expresses is amplified by large standard deviations, usually one third to one half of the means. Furthermore, no information was available on TfR numbers inside adult rat hepatocytes.

There are several technical features which render assaying hepatocyte transferrin receptors a difficult task. First, the receptor is sensitive to the action of proteases contained in collagenase preparations (Young and Aisen, 1981; Morley and Bezkorovainy, 1983; Kishimoto and Tavassoli, 1986; Grohlich et al., 1977; Hatton et al., 1983). Second, hepatocytes, unlike reticulocytes, display a large component of nonsaturable transferrin binding which appears to contribute to iron uptake (Cole and Glass, 1983; Page et al., 1984). Third, endogenous ligand, detectable on the plasmalemma of hepatocytes competes against the added ligand. Thereby, occupancy has been proposed as a likely explanation for the small number of receptors which were found in a study on cultured hepatocytes (Sibille, 1986). Given these kinds of obstacles, it seemed desirable to determine the number of hepatocyte transferrin receptors by a method not involving transferrin binding.

8.2 Experimental Procedures

8.2.1 Materials

Antisera to rabbit and mouse IgG were gifts from Dr. J. Gauldie and the monoclonal MRC OX-26 was the gift of Dr. A.F. Williams (Sir William Dunn School of

Pathology, University of Oxford, UK). N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES), protein A from S. aureus (SpA), monensin, aprotinin, collagenase type IV were all obtained from Sigma (St. Louis, MO). Two batches of the enzyme collagenase type IV were used: lot no. 16F-6805 is referred to below as 'Lot 1' and lot no. 45F-6826 is referred to as 'Lot 2'. Epidermal growth factor was purchased from Collaborative Research, Inc. (Lexington, Mass.). Other materials as described in Chapters 2 and 3.

8.2.2 Reticulocytes and Red Blood Cells

Reticulocyte ghosts were prepared as described in Chapter 3. The resultant ghosts were resuspended in PBS and stored at -40 °C until further use. Red blood cell (RBC) ghosts were prepared as already described for reticulocyte ghosts (Chapter 3, section 3.2.5), except that the donor rats were un-treated adults.

8.2.3 Isolation of Hepatocytes

Freshly isolated hepatocytes were prepared following the procedure outlined in Chapter 3 and Appendix CH-3. Viability of the preparations was assessed by exclusion of trypan blue and was better than 90%. There was < 2% contamination with non-parenchymal cells.

8.2.4 Permeabilization of Hepatocytes

The cells were permeabilized with digitonin according to the procedure of Weigel et al. (1983). The treated and non-treated cells were washed in 2 x 40 ml of 1% BSA in PBS, pH 7.4 before use in the study.

8.2.5 Production of Antiserum to TfR

The technique used to isolate the rat transferrin receptor was that described in detail in Chapter 7. A volume of isolated RTf receptor (RTfR) was combined with an equal volume of Bacto Freund's Complete Adjuvant (Difco Laboratories, Detroit, Michigan, USA) and then used to immunize a female New Zealand White rabbit following the procedure of Vaitukaitis (1981). The animal was boosted one month later. Approximately 40 ml of blood was taken one week after boosting. The resultant serum was absorbed with an insoluble mixture of RTf, rat albumin and BSA, that was polymerized by the method of Ternynck and Avrameas (1976). This was done to remove any antibodies in the antisera that could react with RTf and rat albumin. The absorbed antiserum was precipitated at 33% $(NH_4)_2SO_4$ with saturated $(NH_4)_2SO_4$ and the resulting pellet washed twice with 33% $(NH_4)_2SO_4$ in PBS (10,000 RPM for 20 min 0-4°C). The pellet was then resuspended in PBS and dialysed against the same.

This preparation, which is referred to below as 'PAb', was stored at -40°C until further use. Four non-immunized rabbits of the same strain were bled and the resulting sera were treated as described for the antiserum. This material was used to measure non-specific adsorption of IgG to hepatocytes. To remove any aggregates which may have formed during storage, aliquots of the antiserum and serum, used during particular assays, were filtered through a Millipore Millex-GS sterilizing filter unit with a 0.22 um membrane just before use. IgG concentrations were determined by quantitative immunoelectrophoresis according to the method of Weeke (1973).

8.2.6 Receptor Binding Assays

The binding assays were performed in 1.5 ml Eppendorf microtubes under three different conditions: 1) with ^{125}I-RTf (at a single saturating concentration), 2) PAb or MRC OX-26 - herein after referred to as 'MAb' - (PAb and MAb used at saturating concentrations which were previously determined) and 3) RTf and PAb or MAb. Each of these conditions was tested on: a) reticulocyte ghosts (equivalent to 350-620 ug of cell protein) b) RBC ghosts (equivalent to 350-620 ug of cell protein) c) intact hepatocytes (1-4 x 10^6) and d) digitonin permeabilized hepatocytes (1-4 x 10^6).

All cells were suspended in assay buffer (1% BSA in PBS, pH 7.4) prior to use in binding assays. Triplicates of total binding were performed on the ghosts or hepatocytes. The total volume was 0.4 ml composed of 0.2 ml of hepatocytes or ghosts and 0.2 ml of assay buffer containing the particular protein(s) as defined by the conditions stated above. Non-specific binding of ^{131}I RTf or ^{125}I SpA was determined in triplicate by including a 30-fold excess of human diferric Tf and/or an equal concentration of normal rabbit IgG (mouse IgG in the case of MAb) depending on the assay. All binding assays were carried out on ice and under saturating ligand concentrations. Bound radioactivity was separated from free by centrifugation.

Typically, the ghosts or hepatocytes were incubated with the normal IgG, MAb, PAb or buffer only (for ^{131}I RTf binding) for 1 h with vortexing every 10 min followed by 2 x 1 ml washes with ice cold assay buffer. The pellets were resuspended in the assay buffer to a final volume of 0.4 ml which resulted after addition of the particular labelled ligand. Incubation continued for another hour. In assays where simultaneous RTf and SpA bindings were assessed, labelled SpA was added 15 min prior to the addition of labelled RTf (the order of SpA, RTf addition was inconsequential). Incubations

were terminated by addition of 1 ml ice cold assay buffer and centrifugation followed by a wash with 1 ml of the same. The tips which contained the pellets were cut off the microtubes and counted in 12 x 75 mm glass culture tubes in a Packard model 5986 multichannel analyser.

In binding studies on hepatocytes where the effects of monensin or EGF on receptor expression were examined, the cells were first suspended in Williams E medium which contained 20mM HEPES, 10mM $NaHCO_3$ and 1% BSA, pH 7.5. Those cells incubated at 37°C were prewarmed at 37°C for exactly 10 min before the experiment was begun and incubation was carried out under an atmosphere of 95% O_2 5% CO_2 with swirling at 190 rev/min. Incubations were stopped by addition of 20 ml of ice cold assay buffer and the cells were washed in 2x40 ml of the same (1000 RPM for 1 min at 4°C).

Protein concentrations of ghost preparations were determined according to the method of Lowry (1951) and SpA was quantified according to a procedure described by Sjöquist et al. (1972).

8.2.7 Proteins and Other Procedures

Human transferrin was prepared as described before (Regoeczi et al., 1979). The major component of rat transferrin which is present in the serum i.e.

RTf-3, was used in this study and was prepared as described in Chapter 2. Radioiodinations were carried out using chloroglycoluril as described in Chapter 2. The light-density subcellular fraction from adult rat liver homogenates was prepared by a three-step procedure involving differential and density gradient centrifugations, and gel filtration according to the method described by Debanne et al. (1984). The light fraction was run on polyacrylamide gels according to the technique of Laemmli (1970). Western blotting onto nitrocellulose (BioRad, Mississagua, Ontario) was according to Burnette (1981).

8.3 Results

8.3.1 Verification of Antiserum Specificity

The antiserum to RTfR, PAb, and monoclonal MRC OX-26, MAb, were tested in parallel for reactivity to the light-density vesicular fraction prepared from adult rat liver which is known to be enriched in TfRs (Debanne et al., 1984) which was on a Western blot. The major band of recognition seen in Figure 8.1 corresponds to a molecular weight of approximately 93 KDa. The fainter band, appearing most significantly in lane B, corresponds to an apparent molecular weight of 186 KDa. Other, still fainter bands of recognition seen in both lanes,

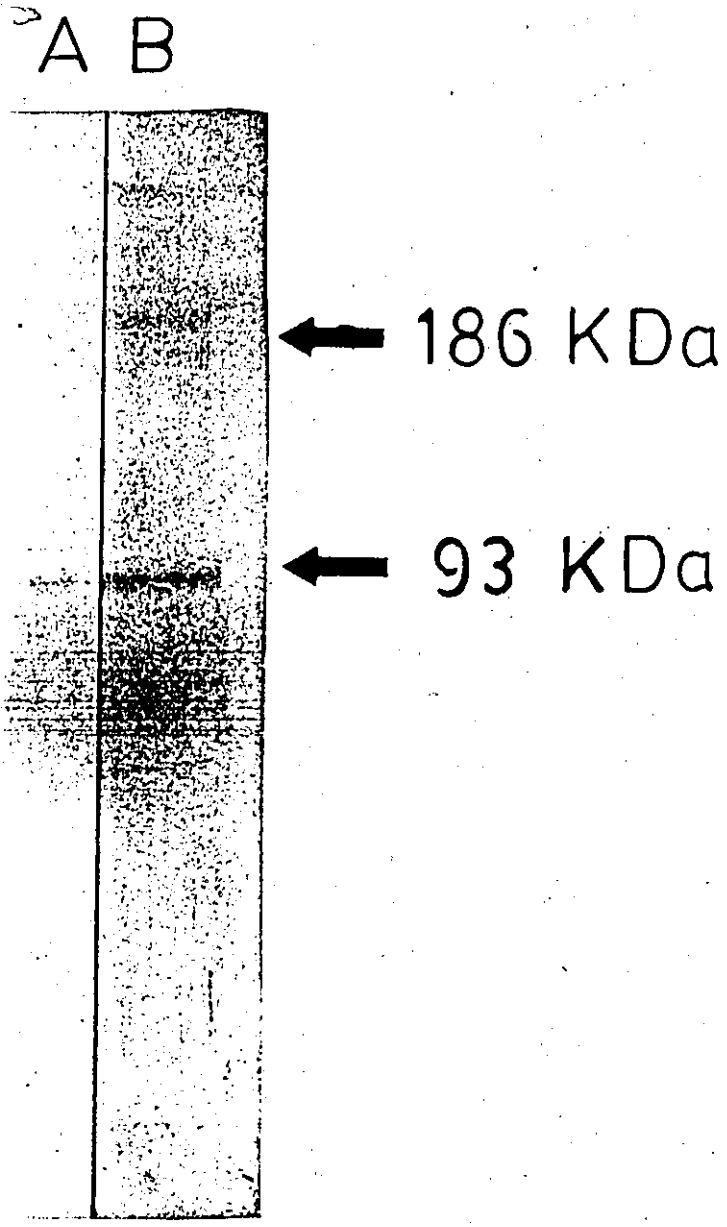


FIGURE 8.1

FIGURE 8.1 Western blot onto nitrocellulose of the light-density subcellular fraction from adult rat liver homogenates which was run on a polyacrylamide gel (see Experimental Procedures, section 8.2.7). Lane A corresponds to identification of protein with MRC OX-26, MAb and lane B represents materials identified by PAb. See text for further details.

are presumed to be proteolytic breakdown products of the receptor and were routinely seen with this type of preparation, but not with preparations of reticulocyte ghosts assayed in a similar manner. Both the PAb and the MAb recognized only the purified rat TfR and the TfR from solubilized reticulocytes on Western blots (not shown). It is therefore assumed that the bands of recognition from the liver tissue are TfR. It could be argued that MAb and PAb might only be able to recognize receptors, which may be predominantly denatured, on nitrocellulose paper. In order to determine whether PAb and MAb were able to bind to non-denatured transferrin receptors, PAb and MAb were tested against reticulocyte ghosts which are known to contain TfR. Red blood cells, on the other hand, are known to contain few, if any TfR (Pan and Johnstone, 1983) and were therefore used as the control cells. Binding of rat Tf to both types of cells served as a control for the presence of competent TfR. The binding results with rat Tf, presented in Figure 8.2, demonstrate the presence of competent TfR on the reticulocyte ghosts but not on the RBC ghosts. The patterns of binding seen with PAb and MAb are similar to that of rat Tf and indicate that they were capable of recognizing TfRs <u>in vitro</u>. In order to determine whether rat Tf interfered with the ability of PAb or MAb to bind to

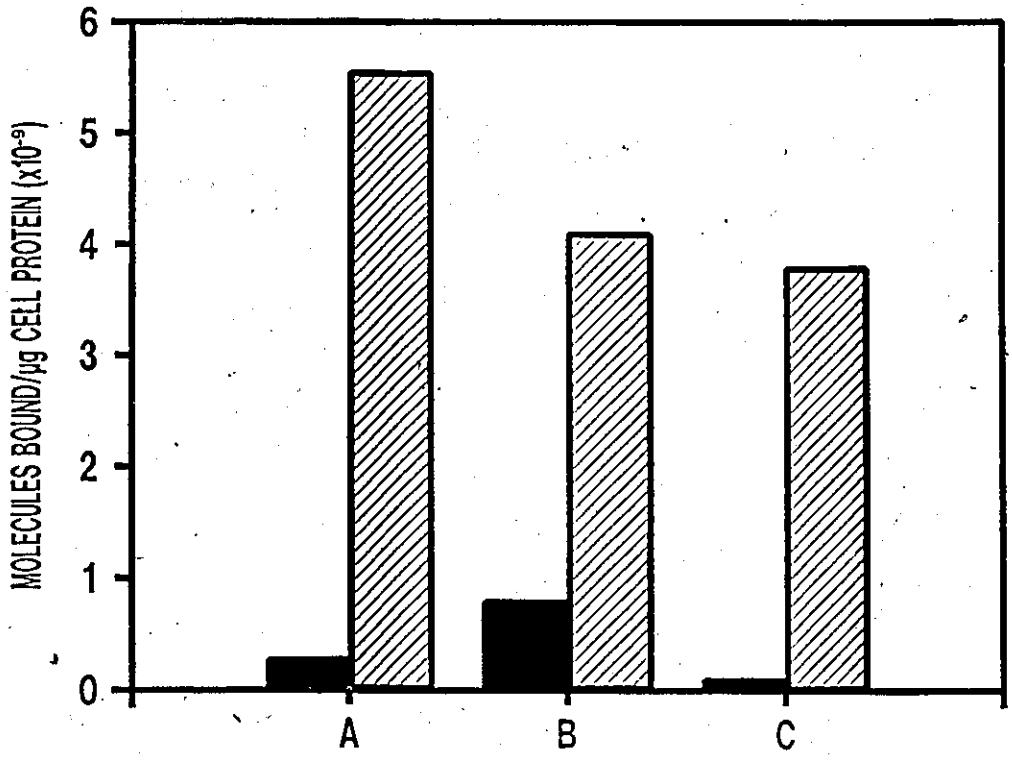


FIGURE 8.2

FIGURE 8.2 Comparison of the binding of MAb (A), PAb (B) and rat Tf (C) to rat reticulocyte ghosts (hatched columns) and rat erythrocytes (filled columns). Radioiodinated SpA was used to trace bound immunoglobulins according to the methods described in Experimental Procedures (section 8.2.6). Approximately 360 ug of cell protein was used per assay.

receptors or vice versa, a final assay was conducted with reticulocyte ghosts. The data (not shown) indicated that polyclonal antibodies did not interfere with Tf binding and vice versa. However, there was a subtle decrease in binding of MAb by Tf bound to the receptor (~14%), whereas there was no effect of MAb on Tf binding.

8.3.2 Quantification of Receptors on Hepatocytes

Freshly isolated hepatocytes were assayed for cell surface TfR. Using 0.055% digitonin to permeabilize the cells (Weigel et al., 1983), the total number of internal receptors accessible to PAb and MAb were also determined. The results are presented in Table 8.1.

Two different collagenase preparations were used and the striking difference in receptor number estimates found on cells prepared with the different lots (due to the untoward effects of the enzyme preparations on TfRs), as estimated by both PAb and MAb, reflected this difference: Collagenase 'Lot 2' left on the cell surface only 19.7% of all detectable receptors i.e., surface and interior, as estimated by MAb and 20.2% using PAb. On the other hand, 'Lot 1' which was found to be less destructive, left on the cell surface, 36.9% of the cell's total number of receptors (estimated by MAb) and 38.9% of the receptors (estimated by PAb). This effect

TABLE 8.1. Estimation of transferrin receptors on hepatocytes using ^{125}I SpA in conjunction with MAb and PAb, and two different preparations of collagenase

Collagenase	MAb			PAB			PAb/MAb	
	Surface	Total	n	Surface	Total	n	Surface	Total
Lot 1	47.7± 8.7	129.2 ± 24.5	5	290.0± 48.2	745.8± 55.2	5	6.1	5.8
Lot 2	14.2 ± 3.6	72.4 ± 12.7	3	55.1± 3.8	272.1± 34.9	12	3.8	3.7

Values, expressed as TfR x 10^{-3} per cell, are means ± SE from n experiments.

of collagenase was emphasized by the results obtained from a liver perfusion where digestion with 'Lot 2' enzyme was extended by 15 min in a single experiment. Effectively no TfR were detectable by ^{125}I Tf binding while MAb and PAb indicated 5,900 and 15,800 receptors respectively.

Data from 16 hepatocyte preparations were subjected to analyses of the variances (Ryan et al., 1985). As is apparent from the F values listed in Table 8.2, and as was expected from the results already presented, the results show marked differences in detectable TfR among the cell preparations and between the two batches of collagenase used.

A significant difference is evident in Table 8.1 with respect to the number of receptors estimated by the two immunological probes. The following pertains to its explanation. It has been shown, that on average only one molecule of SpA binds per molecule of cell bound IgG (Gruhn and McDuffie, 1979). In the case of MAb, because monoclonal antibodies recognize only one determinant per antigen, it was assumed that each molecule of SpA bound represented one receptor. Given that SpA is comparably reactive against the rabbit IgG of PAb and the mouse IgG 2a of MAb (Langone, 1982a), it was calculated that, depending on the preparation of

TABLE 8.2 Analysis of the sources of variations in hepatocyte transferrin receptors

Source	S.S.	DF	V	F
Total	3.275×10^{11}	47	-	-
Cell Preparations	2.217×10^{11}	14	1.584×10^{10}	125.4
Collagenases	1.017×10^{11}	1	1.017×10^{11}	805.2
Experiments	3.235×10^{11}	15	-	-
Residue	4.043×10^{8}	32	1.263×10^{8}	

The abbreviations are: S.S., sum of squares; DF, degrees of freedom; V, variance; and F, variance ratio. For explanations see text.

hepatocytes, approximately 4-6 molecules of PAb rabbit IgG were probably bound to each transferrin receptor in hepatocytes. Division of the receptor numbers quoted for 'Lot 1' in Table 8.1 with PAb by a factor of 6 reveals a result comparable to that with MAb (MAb:PAb - 129,200:124,300 total and 47,700:48,300 surface). Furthermore, the average ratio of surface receptors to internal receptors indicated with PAb was 1:1.89, in comparison to 1:2.07 with MAb.

TfR estimates with ^{131}I Tf were made on the same hepatocyte preparations as those used for the immunological studies. Since the number of cells available from a single liver was limited, Tf binding was done by using a single saturating concentration of ^{131}I Tf. The total receptor number was then calculated from the ratio of added and bound ^{131}I Tf by reference to a Scatchard plot established in a separate study. A value of 15,900 TfR/cell (± 900 SE) was obtained in seven experiments on cells prepared with 'Lot 2' collagenase. When this value is compared with the 14,200 obtained with MAb, as indicated in Table 8.1, it seems to suggest that receptor occupancy was not a problem in these estimations on cells prepared under these conditions.

8.3.3 Receptor Quantification in the Presence of Monensin or EGF

In one preparation of hepatocytes and in two other preparations of hepatocytes, all isolated with 'Lot 1' collagenase, the effects of monensin and EGF, respectively, on Tf receptor distribution were examined. The results are presented in Tables 8.3 and 8.4 respectively. The hepatocytes were divided into three equal groups of cells: 1) control cells which were left on ice 2) control cells which were incubated at 37°C and 3) cells which were incubated at 37°C with either 10 uM monensin or 0.17 mM EGF.

In agreement with a previous report (Kishimoto and Tavassoli, 1986) there was a trend among cells incubated at 37°C to express more surface receptors and this is seen in the results in both tables. The monensin treatment lasted 90 min and caused a reduction in the number of surface receptors by 40% as determined by PAb and 32% by MAb.

The incubation time of 5 min with 0.17 mM EGF was based on the conditions outlined by Wiley and Kaplan (1984) and Davis and Czech (1986). The results as indicated in Table 8.4 were conflicting: in experiment E1, TfR's on the surface were reduced by 25% while they were increased by 37%-59% (PAb and MAb respectively) in experiment E2.

TABLE 8.3 Effect of monensin on TfR distribution in suspended rat hepatocytes

Condition	TfR/cell ($x10^{-3}$) using MAb			TfR/cell ($x10^{-3}$) using PAb		
	Surface	Inside	Total	Surface	Inside	Total
0-4°C, no monensin	38.3	62.5	100.8	265.1	401.6	666.7
37°C, no monensin	43.9	101.4	145.3	477.4	480.8	958.2
37°C, monensin	29.8	100.8	130.6	284.3	480.6	764.9

Final concentration of monensin was 10 uM. Collagenase 'Lot 1' was used to prepare the cells. For further explanations see the text.

TABLE 8.4 Effect of EGF on TfR distribution in suspended rat hepatocytes

Experiment	Condition	TfR/cell (x10^{-3}) using MAb			TfR/cell (x10^{-3}) using PAb		
		Surface	Inside	Total	Surface	Inside	Total
E1	0-4°C, no EGF	N.D.	N.D.	N.D.	267.6	428.1	783.7
	37°C, no EGF	N.D.	N.D.	N.D.	353.0	312.6	665.6
	37°C, EGF	N.D.	N.D.	N.D.	264.1	470.7	734.8
E2	0-4°C, no EGF	51.2	81.1	132.3	274.3	337.3	611.6
	37°C, no EGF	41.1	118.4	159.5	354.2	258.9	613.1
	37°C, EGF	65.4	54.2	119.6	486.5	150.9	637.4

N.D., not determined. Final concentration of EGF was 0.17 mM. For further explanations see the text.

Insight concerning the impact of temperature on the distribution of receptors and availability to the immunological probes in the suspended hepatocyte was coincidentally afforded through these experiments with monensin and EGF. The changes, as expressed by PAb/MAb ratios, are presented in Table 8.5. When the temperature was increased to 37°C the ratio was significantly increased for surface TfR and was reduced for interior receptors but with little overall change in the total ratio. The shift was relatively unaffected by monensin but was attenuated somewhat by EGF.

8.4 Discussion

Estimates of TfR numbers on hepatocytes have traditionally been accomplished through binding studies with Tf and subsequent Scatchard analysis of the data. The determinations performed on hepatocytes either in suspension or in culture, have suggested that there may be from as few as 5,000 (Sibille, 1986) to as many as 66,000 (Young, 1981) surface receptors per cell. There have been no reports on receptor numbers inside adult rat hepatocytes. However, in a Tf binding study on HEP G2 cells, a hepatoma cell line, (Ciechanover et al., 1983) it was found that there were 50,000 surface and 100,000 internal (assessed in the presence of Nonidet P-40)

Table 8.5 PAb/MAb ratios calculated for hepatocytes under different conditions

Condition	PAb/MAb ratio		
	Surface	Inside	Total
0-4°C	6.1	5.3	5.6
37°C	9.7	3.4	5.2
37°C, monensin	9.5	4.7	5.8
37°C, EGF	7.4	2.8	5.3

Values in this table were calculated from data in tables 8.3 and 8.4.

receptors. The present data suggest that there is a total of approximately 1.3 x 10^5 TfR per hepatocyte with about 37% of these expressed on the surface (Table 8.1). While these findings for the cell surface numbers fall comfortably into the wide range of values found in the literature, the present estimates also demonstrate a considerable range. A variety of factors likely responsible for the size of these ranges includes: 1) presence of high and low affinity sites (the low affinity sights could account for the range in values mostly with respect to estimations by PAb); 2) availability of additional determinants upon digitonization of the cells; 3) effects of collagenase on the cells; 4) availability of detectable receptors and 5) presence of contaminating non-parenchymal cells. The most significant of these factors is probably the process of liver digestion with collagenase.

8.4.1 Effects of Collagenase

The deleterious effects of collagenase preparation on hepatocytes is well known (Crane & Miller, 1974; Solyom et al., 1972; Hatton et al., 1983; Kishimoto and Tavassoli, 1986). The untoward effects of different 'Lots' of this enzyme preparation used in these experiments are captured statistically in Table 8.2. In

the separate experiment where the effect of increased exposure to collagenase on hepatocytes was tested by increasing the liver digestion time from 10 min to 25 min, the resulting number of surface receptors detected by Tf was negligible. In the same preparation of hepatocytes, MAb was able to identify 5,000 surface receptors while PAb indicated 15,000 receptors. These findings raise two related issues. First, that some antigenic determinants of the receptor probably remain on the plasmalemma after treatment with the collagenase. Second, because PAb is polyclonal it is likely that more of these receptor determinants are affected by the collagenase than the single determinant of MAb. Indeed, the lower PAb/MAb ratios seen with 'Lot 2' collagenase, support this view.

The cause for the destruction of receptors is generally attributed to the trypsin-like activity (which varies from lot to lot) present in the collagenase. Referring to Table 8.1, it can be seen that the two different lots of collagenase used in these experiments yielded significantly different results with respect to determinable receptors. Some investigators (Crane & Miller, 1974; Grohlich et al., 1977; Young & Aisen, 1980) have used a constant amount of soybean trypsin inhibitor (STI) in an effort to block this activity; however, it

has been clearly demonstrated that the successful isolation of hepatocytes is dependent upon the trypsin-like activity and clostripain present in the collagenase (Hatton et al., 1983; Gallai-Hatchard and Gray, 1971). Therefore it is reasonable to assume that hepatocytes prepared in the presence of STI are still suffering from the "trypsin-like" effects of the collagenase. This assumption is well founded. Examination of the results from a single investigator through a profile of three publications (Young and Aisen, 1980; Young and Aisen, 1981; Young et al., 1983) reveals a range in estimates from 20,000 to 63,000 receptors per cell. Given that the standard errors within each paper (where available) are not as great as this range, it is probable that different lots of collagenase (and therefore different amounts of trypsin-like activity) were used in each study.

8.4.2 Low Affinity Receptor Sites

Various groups (Cole & Glass, 1983; Page et al., 1984; Sibille et al., 1982) have presented data which support the idea that in addition to high affinity TfR, there may be a large number of low affinity sites. It is possible that these sites of lower affinity share some antigenic determinants with the high affinity TfR.

Depending upon the degree of sharing as well as the success of such determinants to survive the collagenase, a range of potential interactions between antibody and low affinity sites could be expected. However, other than by attempting to purify hepatocyte material retained by an affinity column of anti-TfR antibody, there is no easy way to test this possibility or estimate its relative importance in producing variations in available recognition sites.

8.4.3 Receptor Availability

Although receptor occupancy by endogenous Tf was not a contributing factor to variability of receptor estimates in this study (due to the fact that Fe was being chelated with Desferal as early as during liver perfusion), the availability of detectable TfR may have been. It can be seen from data presented in Table 8.3 that incubation of hepatocytes at 37°C resulted in the appearance of "new" internal and external receptors. Weigel et al. (1983) similarly observed an increase in detectable surface asialoglycoprotein (Gal/GalNAc) receptors after incubating hepatocytes for 45 min at 37°C. They did not indicate an increase of internal receptors. These new Gal/GalNAc receptors were found not to have resulted from a shift of an internal pool of

receptors, detectable in the presence of digitonin. Furthermore, in the presence or absence of cycloheximide, Weigel and his colleagues obtained identical results, which suggested that de novo protein synthesis was not responsible. In the light of this finding, protein synthesis was probably not a cause of the results reported here either. Taken together, these results suggest that Tf and Gal/GalNAc receptors probably exist in a digitonin insensitive compartment(s) which is(are) therefore low in their cholesterol content and that the compartment(s) is(are) able to release their receptors given the appropriate conditions (eg. incubation at 37°C for extended periods). What determines receptor distribution to the compartment(s) is unknown, as is the relative quantity of receptors which may be contained in them at the time of cell isolation.

8.4.4 Contamination with Non-Parenchymal Cells

A final factor possibly influencing estimates of TfR, which has only received limited attention (Kishimoto and Tavassoli, 1985), is that of contaminating non-parenchymal endothelial cells. It has been suggested that a preparation of hepatocytes contaminated with as little as 2-3% endothelial cells is enough to account for observed variations in receptor numbers. This does not

seem a likely explanation in the case of our results, however, given that the level of contamination was consistently less than 2%. As well, these findings indicated that lengthened incubation in collagenase decreased detectable surface receptors (see Results) according to the same authors in a later publication (Kishimoto and Tavassoli, 1987) endothelial cell TfR are resistant to collagenase.

8.4.5 MAb versus PAb as a Probe for TfRs

Clearly, of the two types of antibody probes used, the monoclonal is superior. The combining ratio of approximately 4-6 molecules of PAb per molecule of receptor compares well with data presented by Morris and Williams (1975) who showed that up to four molecules of polyclonal horse anti- rabbit IgG can bind to one molecule of rabbit IgG adsorbed on thymocytes. The comparability of data becomes apparent when the different molecular weights are taken into account (IgG, 150,000; TfR, 186,000). It would be possible to standardize each antiserum with a monoclonal antibody; however, this approach would be limited given the changes in PAb/MAb (Table 8.5) observed to occur with changes in temperature.

8.4.6 Effects of Monensin and EGF on Receptor Distribution

Given that for the first time, it was possible to determine TfR distribution in adult rat hepatocytes, it was decided to perform preliminary studies on the effects of two compounds (monensin and EGF) known to affect receptor distribution in other systems. Monensin, a univalent cationic ionophore, has been shown to disrupt the transport of membrane vesicles from the Golgi apparatus to the plasma membrane in eukaryotic cells (Johnson & Schlessinger, 1980), to inhibit the secretion of various glycoproteins (Oda et al., 1983) and the recycling of insulin receptors in hepatocytes (Whittaker et al., 1986) and also to inhibit recycling of human transferrin in K562 cells (Stein et al., 1984). Our results indicate that monensin is also capable of decreasing the number of surface receptors in adult rat hepatocytes, presumably by blocking recycling (Table 8.3). In contrast, the addition of EGF to hepatocytes in suspension gave varying results (Table 8.4). Wiley and Kaplan (1984) and Davis and Czech (1986) found that addition of EGF to fibroblasts caused a transient but marked (doubled and tripled, respectively) increase in the expression of TfR on the cell surface after only 5 minutes. Such a response does not appear to occur in

adult rat hepatocytes. However, EGF has been implicated in causing increased TfR in regenerating rat liver cells (St.- Hilaire and Jones, 1982), but these changes are noted 18-24 hours after partial hepatectomy (Hirose-Kumagai et al., 1984; Tei et al, 1984.) Most work with EGF and hepatocytes suggest that its effects (usually DNA synthesis is studied) become detectable only after 24 hours and in the continued presence of EGF (St. Hilaire and Jones, 1982; Parzefall et al., 1985).

8.4.7 Conclusions

The finding of approximately 1.3 x10^5 TfR per hepatocyte is likely an underestimate of actual TfR numbers given the effects of collagenase and the existence of digitonin-insensitive compartments. However, it does represent the most objective determination of total TfR content in hepatocytes thus far and establishes the realistic potential of an hepatocyte's store of TfR available for recruitment in order to take up diferric Tf. Young et al. (1981) have reported that adult hepatocytes when incubated in the presence of 0.2 mg/ml of rat diferric Tf take up 3.764 x10^4 atoms of iron/min/hepatocyte. Based on the assumumption that each Tf molecule donates two atoms of iron and that each receptor binds only one molecule of Tf, 1.882 x 10^4 receptors/min/ hepatocyte would be necessary to account for the uptake.

If, on average, the cycling time of the TfR is 4 min, a total of 7.528×10^{4} receptors would need to be recruited and this certainly appears to be within the limits of an hepatocyte's resources. By increasing the incubating concentration of Tf to 5 mg/ml, 2.33×10^{5} atoms of iron/min/cell are observed to be uptaken (Young et al., 1981). With a 4 min cycling time this would require the hepatocyte to circulate 4.66×10^{5} receptors, a number significantly larger than it probably possesses. Even reducing cycling to 2 min would require every available TfR. Clearly, iron donated to the adult hepatocyte by Tf must also enter by some alternative mechanism(s) as suggested by Cole & Glass (1983), Page et al. (1984) and Sibille et al. (1982).

It is emphasized here that the implications of this study, alluded to above, do not negate a role for the TfR in iron uptake from Tf by adult hepatocytes. Rather, they serve to clarify its role, in that the TfR is just one important component in what must be seen as a multicomponent uptake system. Further studies to delineate and characterize what is interpreted and suggested as another major component, forms the subject of the next chapter.

CHAPTER 9

UPTAKE OF IRON FROM TRANSFERRIN BY SUSPENDED HEPATOCYTES: THE SIGNIFICANCE OF PINOCYTOSIS

9.1 Introduction

As stated in Chapter 8, the total number of TfR present in digitonin permeabilized adult rat hepatocytes is approximately 140,000 per cell of which approximately 40% are expressed on the surface. Assuming iron delivery to hepatocytes from Tf occurs solely by a receptor mediated process, it should then be possible to saturate this uptake mechanism. In experiments to be reported below it was found that as much as a 410% increase in the concentration of plasma differric rat Tf in vivo was unable to saturate the iron uptake mechanism of the liver. Even if all of an hepatocyte's TfRs were recruited to the surface and receptor recycling time was reduced to 1 min, saturation should have occurred.

Sibille et al.(1982), Cole and Glass (1983), Thorstensen and Romslo (1984a), Page et al. (1984) and Trinder et al.(1986) have suggested that the TfR pathway may be only one component (Trinder et al., 1986) in a multicomponent delivery system of iron from Tf and that adsorptive endocytosis, low affinity receptor endocytosis or fluid-phase pinocytosis may also be contributing pathways. However, the relative contributions of these

pathways have not as yet been clearly defined. In particular, fluid-phase pinocytosis has been suggested to account for anywhere from 5% (Trinder et al., 1986) to as much as 20% (Thorstensen and Romslo, 1984a) of the total uptake and these estimates were based on the view that fluid-phase markers are only uptaken and retained by cells (Ose et al., 1980). Yet it is clear that in cells which demonstrate a high rate of endocytosis, like hepatocytes (Wileman et al., 1986), large amounts of surface membrane are internalized and rapidly recycled to the surface in order to maintain cell surface area (Van Deurs and Christensen, 1984). In keeping with this concept, earlier work by Besterman et al.(1981) with macrophages and fibroblasts demonstrated the exocytosis of pinoctosed material. More recent work by Scharschmidt et al.(1986) with cultured rat hepatocytes and perfused rat liver has indicated that inulin, a fluid-phase marker, is endocytosed, but is also mostly exocytosed (~80%) by a rapidly turning-over compartment (T1/2 1-2 min) with only ~18% of the material moving to storage compartments. Clearly, such a system could enable large quantities of material to be uptaken and be quickly returned to the cell surface. Thus, in the light of this work and the uncertainty of the contribution to iron/Tf uptake through pinocytosis, it was decided to re-examine

the potential contribution of fluid-phase pinocytosis to the delivery of iron from Tf to the adult rat hepatocyte. To achieve this objective, the uptake and release of the pinocytosis marker polyvinylpyrrolidone (PVP), from hepatocytes in suspension, as well as that of Tf and iron was studied.

9.2 Experimental Procedures

9.2.1 Materials.

Polyvinylpyrrolidone (PVP), which had an average mol. wt. of 36,000, was a gift from Dr. W. Scholtan (Bayer AG, Leverkusen, Germany). Chloroquine phosphate was obtained from Sterling Drug Ltd., Aurora, Ontario. Other relevant materials were obtained as described in Chapters 2 and 3.

9.2.2 Proteins.

Human transferrin was prepared as described before (Regoeczi et al., 1979), and rat transferrin was prepared as outlined in Chapter 2. Desialylation of fetuin was achieved by incubating 100 mg of the protein at 37°C for 20-24 h in 2 ml of 0.1M Na-acetate buffer pH 6.0 containing 1mM $CaCl_2$ and 0.1% (w/v) sodium azide, with 500 units of neuraminidase in a dialysis tubing all

contained in a 100 ml beaker of the above buffer. The buffer was changed at 1 h and 2 h after beginning the incubation. Separation of asialofetuin, incompletely desialylated fetuin and the enzyme was carried out by chromatography on a DEAE-cellulose column (30 x 1.5 cm) that had been equilibrated with 0.01M Tris-HCl, pH 8.0 and was eluted with 0.2M Tris-HCl, pH 8.0.

9.2.3 Isolation of Hepatocytes

Freshly isolated hepatocytes were obtained by liver perfusion with collagenase as described in Chapter 3 and Appendix CH-3. Viability of the preparations, assessed by the exclusion of trypan blue, was better than 90%. There was less than 2% contamination with non-parenchymal cells.

9.2.4 Studies with Hepatocytes

9.2.4.1 Uptake Studies

Cells (range: 8-12 x 10^6/ml) contained in 20 ml glass scintillation vials were brought to 37°C in either MEM or Hank's balanced salt solution (HBSS) with 1% BSA (w/v), pH 7.4, for ten min before the beginning of any experiment; preincubation periods with chloroquine or NaCN and KF occurred after this 10 min period. "Dose" solutions containing the relevant combinations of

radiolabelled and native ligands in the incubation buffer (see captions to Figures for particulars) were added at zero time and cells were maintained in suspension at 37°C by shaking at 100 strokes/min in a shaking water bath. The final volume of each assay was from 3.4 to 5 ml. Samples (200 ul) were taken from the suspension at selected times either in duplicate or triplicate depending upon the experiment. The samples were then treated as already described in Chapter 6, section 6.2.4.

9.2.4.2 Release Studies

In experiments where the release of radiolabelled ligands from the hepatocytes was followed, cells were incubated under the above conditions in the presence of the "Dose" solution for 15 or 45 min. Incubations were terminated by addition of 30 ml of ice-cold incubation medium. The cells were then centrifuged at 100 g for 1 min (0-4°C), followed by resuspension and two further washes with the same final volume of ice cold medium. The cells were finally resuspended to their initial incubation volume and reincubated at 37°C for the indicated time periods. At 2, 4, 6, 8, 10, and 15 min samples were taken as described above and the cells were then centrifuged at 100 g for 10 sec (0-4°C), the

supernatant removed and cells resuspended in fresh medium to the incubation volume. Final volumes of resuspension were decreased by 1 ml for the 6 and 8 min samples and by 2 ml for the 10 and 15 min samples (to maintain cell numbers/ml approximately constant). Experimental samples were performed in duplicate unless otherwise stated. All samples were assayed in a Packard multichannel analyzer model 5986.

9.2.5 Animal Experiments

Rats (180-300 g, females) were given free access to Purina rat chow and water. Injections of ^{59}Fe labelled Tf doses (2.5 ug, 8.5 mg and 20 mg/100g/animal) were performed on tail veins and included 2-5 ug/100g of ^{125}I-Tf which served to distinguish between ^{59}Fe activity taken up by the liver and that trapped in the hepatic vascular bed post-mortem. (For more details, see Chapter 3, section 3.2.4(II).)

9.2.6 Reactions with ^{59}Fe or ^{55}Fe and Radioiodine

Labelling of RTf with ^{59}Fe and charging RTf and HTf with ^{55}Fe were accomplished as described in Chapter 3. Radioiodinations of the proteins were carried out as described in Chapter 2 using chloroglycoluril.

PVP was iodinated essentially as indicated elsewhere (Regoeczi, 1976) but will be described here

briefly. PVP (100 mg) was dissolved in 5 ml of 0.2M H_2SO_4 on ice. From this 5 ml, a 250 ul aliquot (5 mg PVP) was placed into a 1 ml quartz cuvette. To this was added 25 ul of sodium nitrite (10% w/v) followed by 0.5 mCi of $Na^{125}I$ (12.5 ul) and the total was mixed well with a Pasteur pipette. The reaction was allowed to proceed under ultra-violet radiation (210-280 nm) for 60 min. The reaction was terminated by the addition of 300 ul of 0.2M KOH; this was followed immediately by 50 ul of sodium sulphite (5% w/v). The resulting volume was passed through a Dowex anion exchanger (1 X 4)(in the chloride cycle). The column was then washed with 1 ml of 0.9 % NaCl. The effluent was collected, then dialysed against 0.005M HEPES and 0.01M NaI (4 x 250 ml) over 4h at 4°C. This was followed by dialysis against 4 x 500 ml in phosphate buffered saline overnight at 4°C. Figure 9.1 illustrates the chemical structure of the PVP chain and a site of iodination.

Through the course of these experiments it became relevant to answer the question of whether chloroquine and PVP demonstrated any attractive interaction. This was tested by examining the chromatographic elution profile of radioiodinated PVP and its effect on a baseline concentration of chloroquine in the equilibrating buffer (phosphate buffered saline, pH 7.4). A

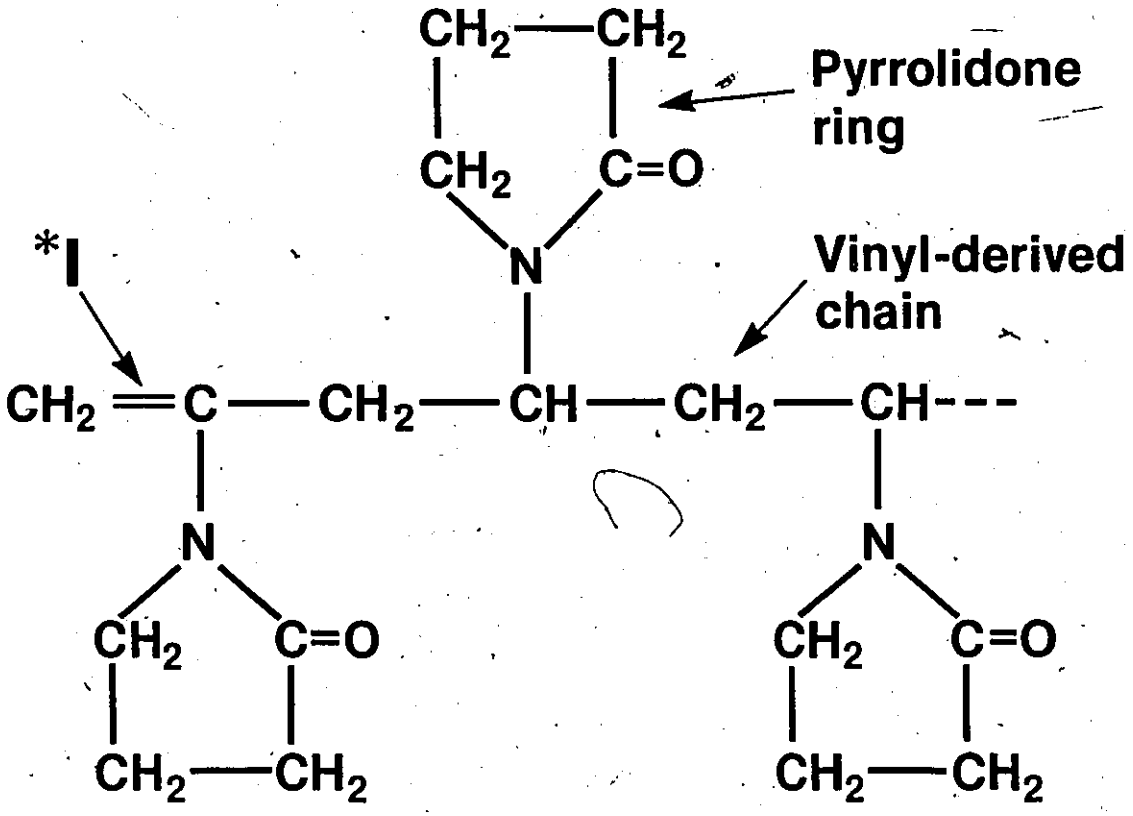

Pyrrolidone ring
Vinyl-derived chain
*I
CH2
C
CH2
CH
CH2
CH
N
C=O
CH2
CH2

FIGURE 9.1

FIGURE 9.1 Chemical structure of polyvinylpyrrolidone (PVP). *I indicates a site of iodination on the PVP chain. See Experimental Procedures (section 9.2.6) for details.

spectrophotometric scan of light absorption by chloroquine revealed an absorption maximum at 341 nm. A concentration of 10 ug/ml of the drug in the equilibrating buffer, gave a reading of 0.20. Following equilibration of a column of Sephadex G-100 with the equilibrating buffer, a 5 mg sample of PVP was loaded and the absorbance of the effluent was monitored in a Beckman DU-40 spectrophotometer.

9.2.7 Evaluation of Uptake and Release Data

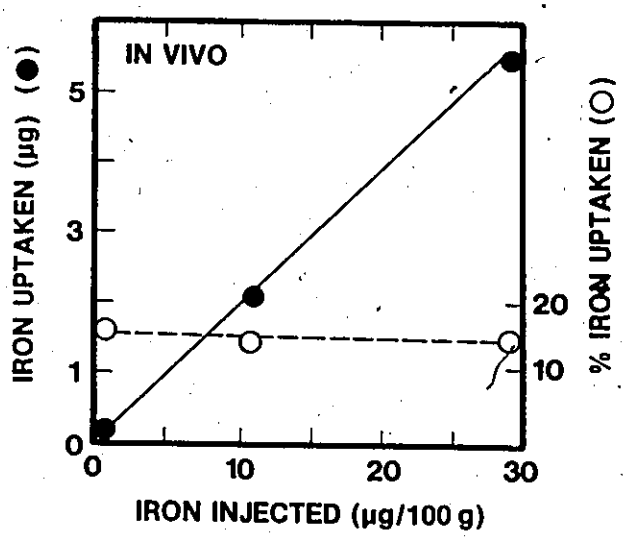
All of the experiments were performed on two to three separate preparations of hepatocytes with points measured in duplicate or triplicate. The experiment in Figure 9.2 was performed on only one preparation of hepatocytes, in duplicate. The data from the uptake and release experiments were assessed by means of the IBM PC version of the statistical software "Minitab" (Ryan et al., 1985). The mean values obtained were then converted into continuous algebraic functions and fitted with three- and two-term exponential equations. The program was based on the computer routine of Nelder and Mead (1965). Deconvolution analysis of the curves resulted in estimates of the total amounts of PVP, Tf and radioiron passing through the cells in suspension with time. The program for deconvolution was designed by Dr. P.A. Charlwood for the main-frame computer of the National

Institute for Medical Research, London, UK. Dr. Charlwood adapted that program to the microcomputer.

9.3 Results

9.3.1 Apparent Non-saturability of Iron Uptake

On the assumption that iron delivery to hepatocytes from Tf occurs solely by a receptor-mediated process, saturation of this mechanism was attempted in these experiments by presenting increasing loads of diferric RTf to the liver *in vivo* and hepatocytes in suspension *in vitro* (Figure 9.2 upper and lower panels respectively). *In vivo* radioiron uptake was examined in rats that were given a single dose of diferric Tf equivalent to 14 to 410% of their circulating Tf (as estimated - see Chapter 4 section 4.3.1). Under these conditions, uptake of iron was linear with a constant percentage of the dose being uptaken independent of the load. *In vitro*, where the range in concentration of diferric Tf was from 2 ug/ml to 1000 ug/ml (500-fold increase), a qualitatively similar result was obtained. Inspection of the lower panel of Figure 9.2 reveals that except for the lowest concentration, the percentage of the load sequestered by the hepatocytes was, as seen *in vivo*, constant over the entire concentration range of


IN VIVO
IRON UPTAKEN (µg) (●)
% IRON UPTAKEN (○)
IRON INJECTED (µg/100 g)
0
1
3
5
10
20
30

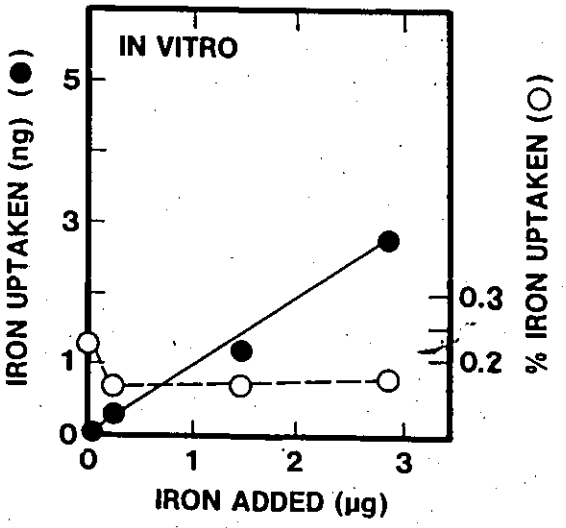

IN VITRO
IRON UPTAKEN (ng) (●)
% IRON UPTAKEN (○)
IRON ADDED (µg)
0
1
2
3
5
0.2
0.3

FIGURE 9.2

FIGURE 9.2 Dependence on concentration of iron uptake from diferric transferrin *in vivo* and *in vitro*. Studies of uptake into the liver (*in vivo* - top panel) were carried out as described in Experimental Procedures (section 9.2.5). Hepatocytes (*in vitro* - bottom panel) in suspension (1 x 10^7/ml) were incubated with 2 to 1000 ug/ml of diferric Tf at 37°C. Sampling and radioactive determinations were performed as described in text. Points represent the means of duplicate samples and are presented as absolute values (filled symbols) and percentages (open symbols).

diferric Tf added. Uptake of a constant percentage of a given load suggests that uptake obeys first-order kinetics both by the liver *in vivo* and by hepatocytes *in vitro* (beyond a critical concentration in the latter case).

9.3.2 Measurement of Fluid-Phase Pinocytosis in Hepatocytes

Since first-order kinetics are not consistent with uptake by receptor mediated endocytosis the findings above prompted evaluation of the pinocytotic rates of heptocytes in suspension. Radiolabelled PVP was used as the most appropriate marker based on the findings of Ose et al.(1980). Initial experiments suggested the presence of a rapid cell-association phase during the early moments of incubation. Given PVP's significant capacity for hydrogen-bonding (De Duve et al., 1974) it is reasonable to believe that the molecule would be present in the coat of medium which necessarily would accompany each cell on its journey through the hydrophobic dibutyl phthalate. Therefore, in order to distinguish between uptaken and surface associated material, studies with the metabolic inhibitors NaCN and KF at 37°C over 15 min were conducted. Confirmation of the inhibitory effects on endocytosis were made by parallel measurement of

asialofetuin uptake - which is attributed solely to receptor-mediated endocytosis - both in the presence and absence of the inhibitors (Figure 9.3). (The association of asialofetuin measured in the presence of the inhibitors, see lower curve in Figure 9.3, can be explained entirely by surface Gal/GalNac receptors.) Under these conditions it was found that 2.2% of the given dose of PVP was surface associated material. All measurements with PVP were therefore corrected by this factor.

In the presence of 1 mM chloroquine, suspended hepatocytes took up more PVP per unit time than cells from the same liver preparation, but without chloroquine (Figure 9.4). On the other hand, the release of PVP was comparable for about the first 80% of the cell-associated material, however, the final 20% was released more quickly in the presence of the drug (Figure 9.5). It is relevant at this point to indicate that the chromatographic experiments with PVP and chloroquine, outlined in the Experimental Procedures, resulted in no demonstrable binding (which might have been responsible for this increased association). The viability of the cell preparations at the end of the experiments with chloroquine, and those described below, was greater than 90%.

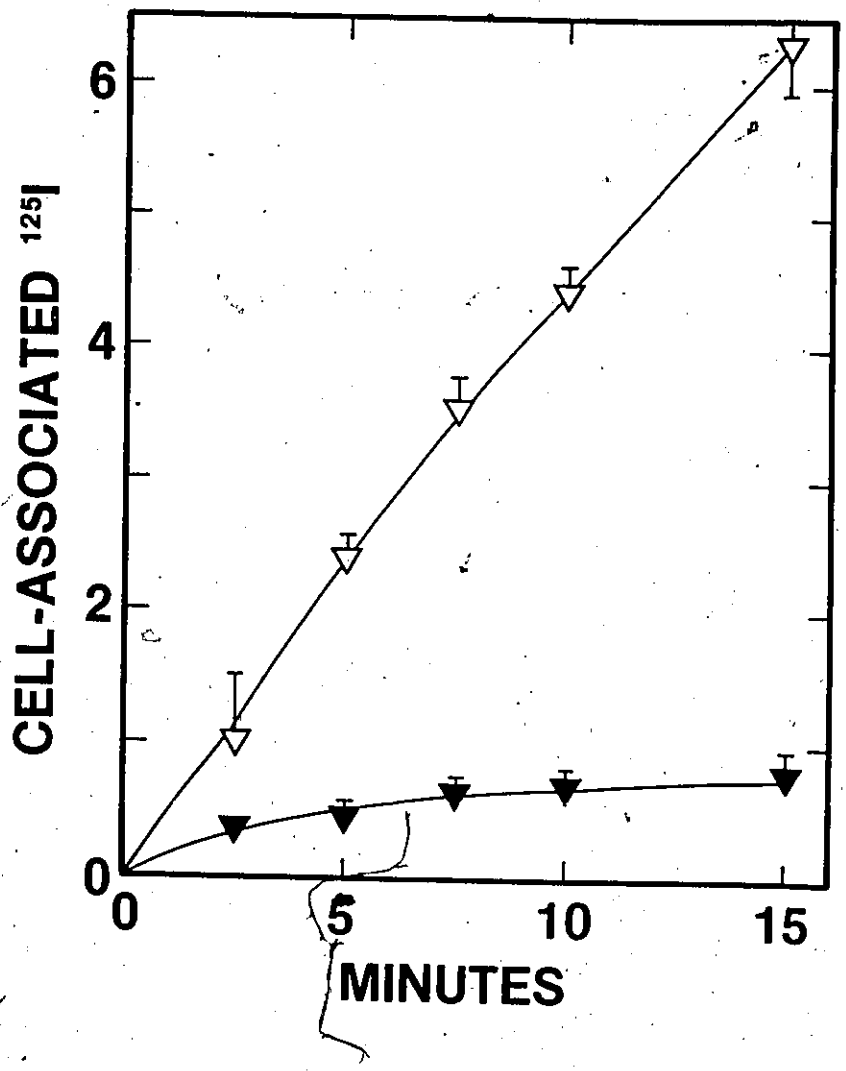


FIGURE 9.3

FIGURE 9.3 Effect of metabolic inhibitors on uptake of asialofetuin by hepatocytes in suspension. Asialofetuin (2 ug/ml) incubated with hepatocytes (1 x 10^7/ml) in the absence (▽) or presence (▼) of 6 mM KF and 10 mM NaCN in the medium at 37°C. Sampling was performed as outlined in Experimental Procedures (section 9.2.4.1). Results are expressed as micrograms of radioiodinated asialofetuin associated with 1 x 10^6 cells. Points represent the means ± SE of four measurements.

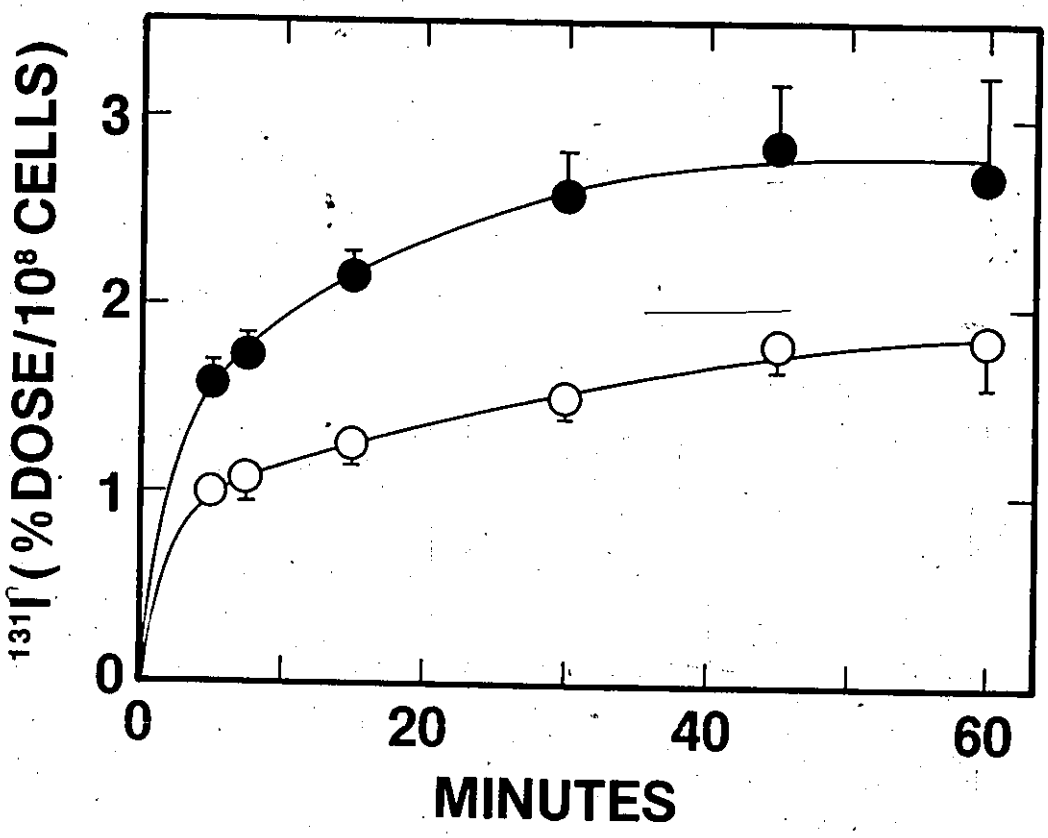


FIGURE 9.4

FIGURE 9.4 Effect of chloroquine on uptake of PVP by suspended hepatocytes. Cells (8-12 x 10^6/ml) and iodinated PVP (200-250 ug/ml) were incubated at 37°C in the absence (○) or the presence (●) of 1 mM chloroquine. Determination of quantities taken up by cells is described in Experimental Procedures (section 9.2.4.1). Points represent the means ± SE of measurements in triplicate from three experiments. Curves were computer-fitted using three exponential terms.

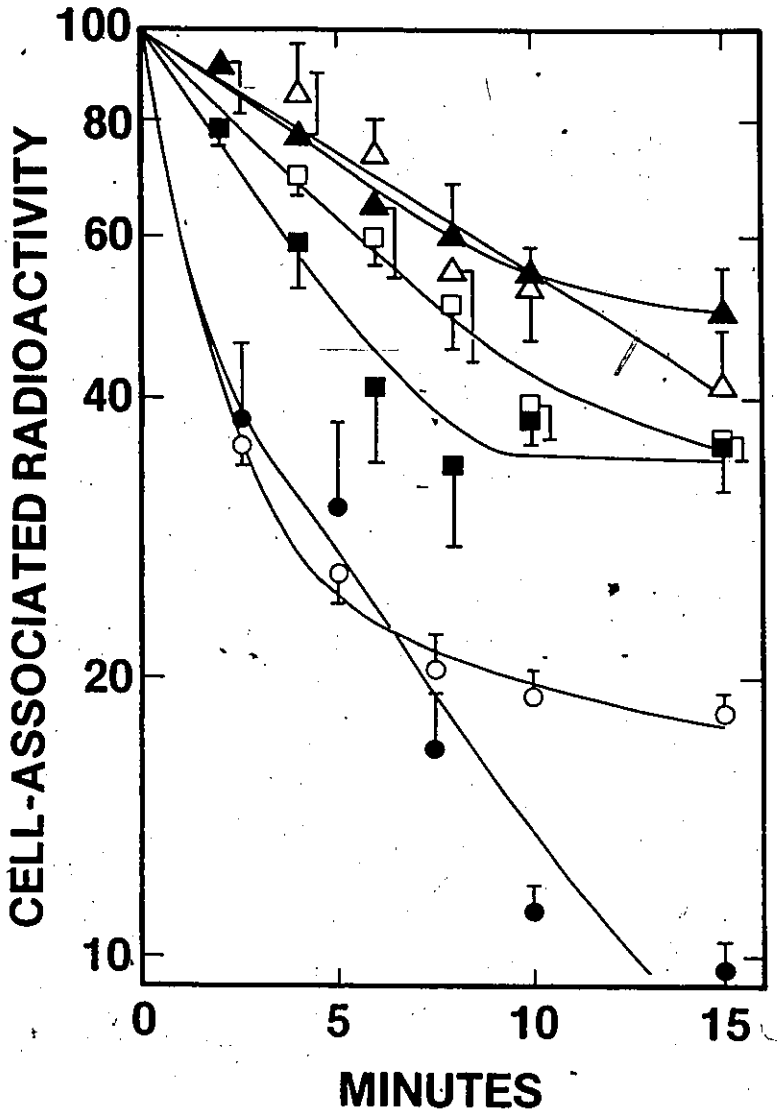

CELL-ASSOCIATED RADIOACTIVITY
100
80
60
40
20
10
0
5
10
15
MINUTES

FIGURE 9.5

FIGURE 9.5 Effect of chloroquine on the release of radioligands from hepatocytes in suspension. Cells (8-12 x 10^6) were incubated without (open symbols) or with (closed symbols) 1mM chloroquine at 37°C. Release of materials was investigated as described in Experimental Procedures (section 9.2.4.2). The following ligand concentrations were used in the initial incubation: ^{131}I-PVP (200-250 ug/ml) (○●); ^{125}I-Tf (2 ug/ml) (□■); ^{59}Fe - presented as diferric Tf (100 ug/ml) (△▲). Preincubation with radio-PVP was for 15 min and 45 min for the other ligands. Results are expressed as percentages of cell-associated material. Points represent the means ± SE and the curves were computer-fitted using two exponential terms.

9.3.3 Uptake and Release of Transferrin and Iron

9.3.3.1 Transferrin

To ensure effective blockade of the Tf receptor, all incubations included 1.55 mg/ml of diferric HTf (due to its 7 fold higher receptor affinity). This represented a 775-fold excess of diferric Tf. Inspite of this, as can be seen from the data presented in Figure 9.6, there was an early, rapid uptake of material followed by a slower rate of accumulation. The Tf uptake effectively plateaued at approximately 0.27% dose/10^6 cells, by 15 to 30 min. Uptake in the presence of chloroquine was slower in the early minutes, reached identity by approximately 25 min and subsequently continued to increase.

The release of Tf over 15 min in the presence of chloroquine amounted to approximately 65% of that initially present and occurred at a higher rate than that observed in the absence of chloroquine. However, by the end of the same time period effectively the same amount of Tf had been released in the absence of the drug (Figure 9.5).

9.3.3.2 Iron

The ability of hepatocytes to uptake iron from Tf in the absence and presence of 1 mM chloroquine was also

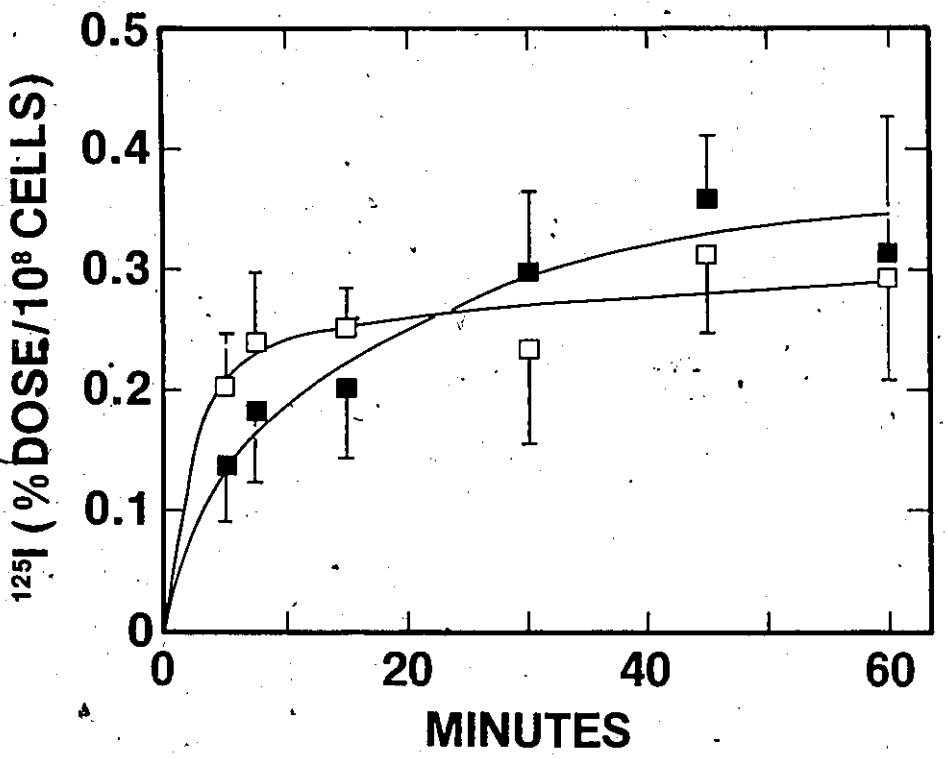


FIGURE 9.6

FIGURE 9.6 Effect of chloroquine on the association of diferric Tf with hepatocytes. Cells in suspension (8-12 x 10^6) with iodinated diferric Tf (2 ug/ml) were incubated in the absence (□) or presence (■) of 1 mM chloroquine at 37°C; the medium contained 1.55 mg/ml diferric human Tf in both instances. Sampling was conducted as described in Experimental Procedures (section 9.2.4.1). Points represent the means ± SE of measurements in triplicate from two experiments. The curves were computer-fitted using three exponential terms.

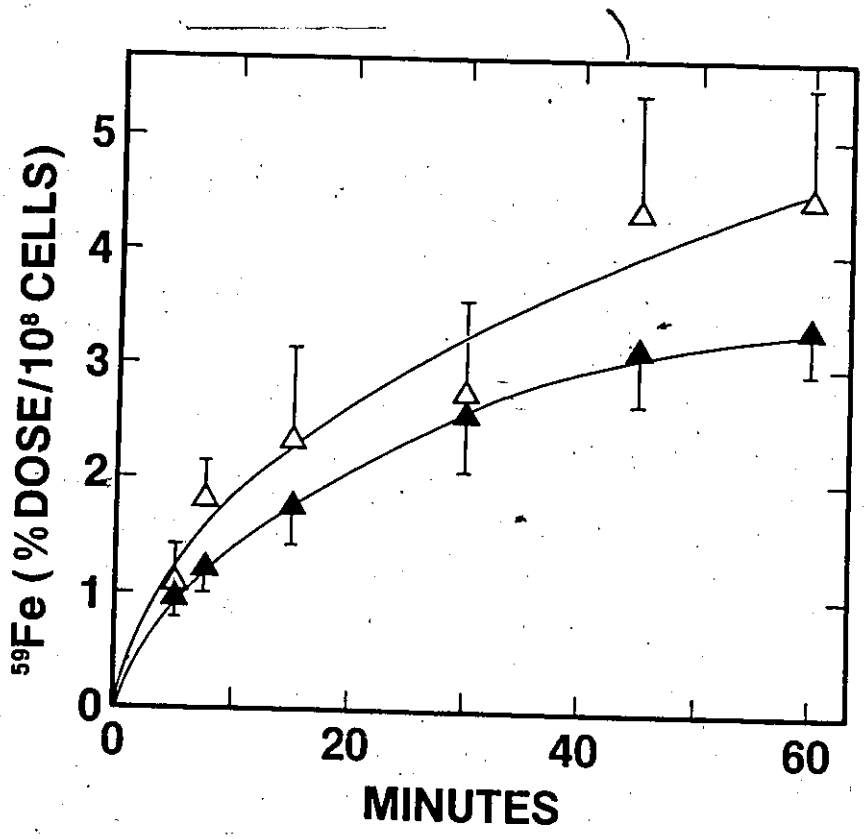


FIGURE 9.7

FIGURE 9.7 Effect of chloroquine on the uptake of iron from Tf by hepatocytes. Diferric ^{59}Fe Tf (100 ug/ml) was incubated with suspended hepatocytes (8-12 x 10^6/ml) in the absence (△) and presence (▲) of 1mM chloroquine. Sampling was performed as outlined in Experimental Procedures (section 9.2.4.1). Points represent the means ± SE of the values obtained in triplicate from two experiments. The results were fitted to three exponential terms by computer.

investigated (Figure 9.7). Verification of the ability of chloroquine to elevate pH was obtained in concurrent experiments in which uptake and production of TCA-soluble (degradation) products from asialofetuin were measured. In the presence of 1mM chloroquine, uptake of asialofetuin was significantly reduced and TCA soluble ^{125}I was negligible when compared to controls (not shown).

Iron uptake in the presence of chloroquine was mildly depressed from the level of uptake observed in the absence of the drug after 60 min of incubation (Figure 9.7). Based on the observations of others (McArdle et al., 1985; Swaiman and Machen, 1986), a greater reduction in uptake was expected.

The release of radioiron from hepatocytes (Figure 9.5) was not significantly different in the presence of chloroquine from that observed under control conditions.

9.3.4 Deconvolution Analysis

Data from the experiments concerned with uptake and release of materials allowed for calculation of the total amount of ligand which passed through the cells and expression of this as an accumulation of materials within the cells as a function of time. In other words, it

became possible to answer the question: If given the hypothetical situation that cells did not exocytose any materials taken up, how much PVP, Tf and iron would hepatocytes accumulate over a given period of time? Deconvolution analysis of the fitted exponential curves from both uptake and release experiments results in a mathematical expression of this relationship. The calculations were performed by computer as outlined in Experimental Procedures, section 9.2.7. These functions are presented graphically in Figure 9.8. Ideally, in the steady state system, the curves would be represented as straight lines, however the results depict the relationships as somewhat non-linear. This is most likely due to the error accrued through the technical difficulties of performing these experiments, particularly with respect to the release studies. Errors would result from the problems of maintaining a constant volume and achieving identical release conditions. The variability of performance, for both uptake and release, between experimental preparations of hepatocytes (as has also been noted by Scharschmidt et al. (1986) in similar kinds of experiments) is also a significant factor which would contribute to the variability of the data. However, given that there is not a great divergence from linearity it is reasonable to make the following

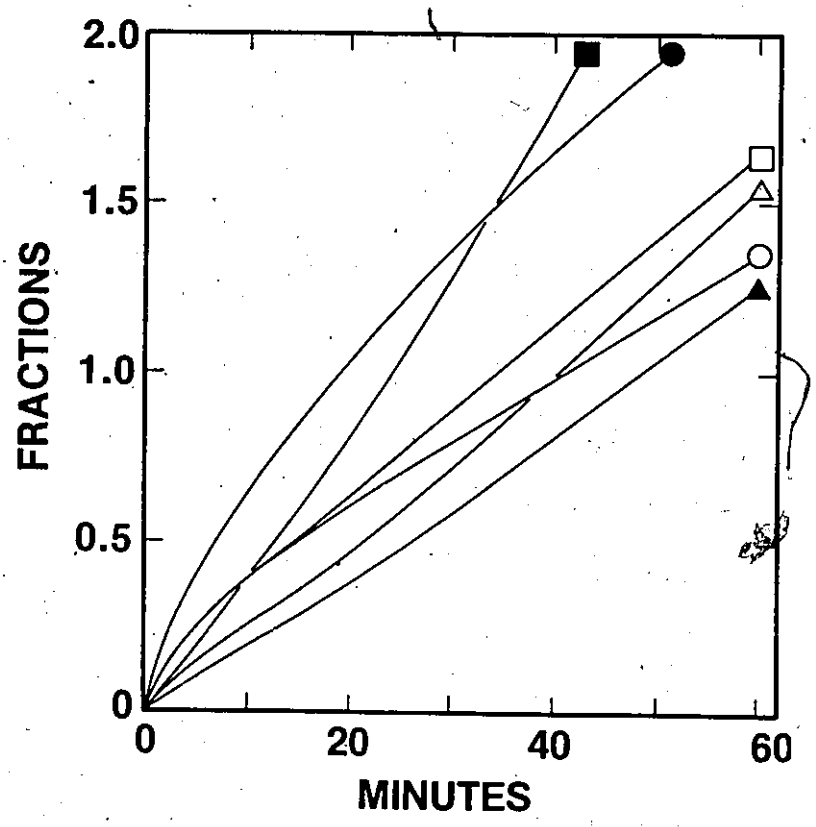


FIGURE 9.8

FIGURE 9.8 Total accumulation of PVP (○●), Tf(□■) and Iron-Tf (△▲) by hepatocytes as revealed by deconvolution analysis in the absence (open symbols) and presence (closed symbols) of chloroquine. Uptake data for these three ligands as presented in Figures 9.4, 9.6 and 9.7 respectively coupled with corresponding release data from Figure 9.5 were subjected to computer analysis as outlined in Experimental Procedures. The results from the data analysis were normalized to 1 x 10^{6} cells/ml with the values for the ordinate being scaled to fractions of PVP, Tf or iron-Tf in the medium.

observations from these curves. First, it is apparent that PVP, Tf and iron are comparably accumulated by hepatocytes in the absence of chloroquine according to computer analysis. Second, the accumulation of iron from Tf is reduced in the presence of chloroquine. Third, a remarkable increase in the accumulation of both PVP and Tf occurs in the presence of chloroquine compared to their respective accumulations in the absence of the drug (compare the filled-in circle and square with the open circle and square, respectively). It can also be seen that there is almost identical accumulation of both PVP and Tf in both the absence and presence of the drug.

9.4 Discussion

Radioiron uptake data from the experiments _in vivo_ and _in vitro_ (Figure 9.2, upper and lower panels, respectively) are consistent with the proposition that the major route for iron accumulation in the liver is through pinocytosis. (The consistent uptake observed in experiments with iron nitrilotriacetic acid (Fe-NTA), as was reported in Chapter 5, further underlines this proposition.)

In vivo data in these experiments indicate that ~20% of the iron from diferric Tf becomes liver associated in 2 h regardless of the load of diferric Tf

presented. This implies that at the highest concentration tested, approximately $8x10^{16}$ molecules of Tf would have to have been in contact with the hepatocytes in order to donate the iron. If it is assumed that a typical 200 g rat's liver weighs 10 g and contains $1.25 x10^{9}$ hepatocytes (Manthe-Kaas et al., 1976) and if all of an hepatocyte's Tf receptors were involved in Fe uptake from Tf, i.e., ~140,000/cell (Chapter 8), then $1.75x10^{14}$ receptors would be available in a typical rat's liver. Receptor recycling times for the Tf, TfR complex have been estimated at 15.8 min in Hep G2 cells (Ciechanover et al., 1983), 12.5 min in K562 cells (Weissman et al., 1986) and 3 min (Iaccopetta and Morgan, 1983a) and 4 min (Nunez and Glass, 1983) in rabbit reticulocytes. Combining the recycling time from Hep G2 cells (15.8 min) with the aforementioned receptor numbers yields a rate of $3.7x10^{13}$ receptors recycled/ hour/liver. Given the observed uptake ($16 x10^{16}$ atoms of Fe = $8x10^{16}$ molecules of Tf) approximately 60 times more material was found associated with the liver by 2 hours than could be accounted for by receptor-mediated uptake. Even if the receptor recycling time was 3 min, ~11 times more material was present in the liver than accountable for by receptors. The astounding aspect of these revelations is that if we assume that 20% of normal, physiological

levels of circulating Tf is taken up by the liver i.e. no injected material, then there would still be 13 times more or 2.5 times more material in the liver at 2 hours than would be accounted for by receptors assuming 15.8 min or 3 min recycling times, respectively. This point is further emphasized when it is realized that we have been discussing an idealized situation where the total cell surface and all of the TfRs are in contact with the Tf-laden extracellular fluid. However, only approximately 72% of an hepatocyte's total surface area constitutes the plasmalemma (Weibel et al., 1969), and because approximately 60% of the cell's TfRs are intracellular (Chapter 8) all of the receptors of an hepatocyte are probably not simultaneously involved in Tf-mediated iron uptake. It is clear therefore, that pinocytosis is the likely route.

It has been demonstrated by Sibille et al. (1982) and in the present thesis (see Chapter 6) that uptake of diferric Tf at low concentrations (0.05 ug/ml per 1 x 10^7 cells/ml) is probably mediated by TfR. This is also reflected by the finding of a higher percentage of the dose uptaken at comparably lower concentrations as illustrated in Figure 9.2 (lower panel). But, as is clear from the situation *in vivo* discussed above and *in vitro* at higher concentrations of competing diferric Tf, the percentage uptake is a constant and consistent with a pinocytotic mechanism.

Further strong support for the idea that most of Tf-mediated iron delivery to hepatocytes is by pinocytosis is derived from the highly similar accumulation curves observed for Tf and PVP as illustrated in Figure 9.8. It is important to recall that these data reflect non-receptor mediated uptake, given the excess of diferric human Tf present in the incubation media of these experiments. Earlier studies of pinocytotic capacity have been largely based on the assumption that pinocytotic rates are estimated on the basis of cell-associated material (Ose et al., 1980; Thorstensen and Romslo, 1984a; Trinder et al., 1986). The combined results from data in Figures 9.4, 9.6 and 9.7 which give rise to the deconvolution curves in Figure 9.8 demonstrate that these earlier studies significantly underestimated the capacity of this mechanism. This same conclusion was also reached by Besterman et al. for sucrose (1981 and 1983).

Clearly, the validity of attributing the vast majority of Tf uptake to pinocytosis rests squarely on the virtues of PVP as a reliable marker of fluid phase pinocytosis. Indeed, it has been suggested that PVP represents the best marker of just this type of uptake (Ose et al., 1980). However, it has been demonstrated that the uptake of PVP will vary from cell type to cell

type and depends upon the size of the polymer used (Duncan et al., 1981). For such reasons, a polymer stock of consistent size distribution was used throughout these studies. However, given the findings of Duncan et al. (1981) it is possible that the endocytosis of PVP may proceed by some combination of adsorption as well as fluid phase. This situation of "mixed type" uptake (Jacques, 1973) is also probable for Tf especially given the proposed insaturable low affinity sites (see Chapter 8 and Bomford and Young, 1983). This view of mixed-type uptake, coupled with the similarity of accumulation curves demonstrated for both Tf and PVP without and with chloroquine (Figure 9.8) argue in favour of both proceeding by the same mechanisms.

Chloroquine's effect on: 1) Tf and 2) PVP's increased apparent accumulation by hepatocytes, is most likely due to the drug's effects on intracellular transport vesicles. Chloroquine has been demonstrated to cause a number of molecules, such as lysosomal enzymes in fibroblasts (Hasilik and Neufeld, 1980) and de novo synthesized ACTH in pituitary cells (Moore et al., 1983), to be directed into the secretory pathway instead of into lysosomes and secretory granules, respectively. Furthermore, it has been shown to inhibit the conversion of multivesicular bodies to secondary lysosomes (Hornick et al., 1984) with resultant exocytosis of non-degraded

epidermal growth factor (Wakshull et al., 1985). On the basis of such findings it seems plausible that pinosomes which may have been routed to the cellular interior are "short-circuited" back to the cell surface. Such a proposal is consistent with the findings in these experiments (Figure 9.8). Indeed, Casey et al. (1986) have shown by Percol gradient fractionation that pinosomes can be resolved into at least two populations based on their buoyance. They have demonstrated an early and a late population, the latter of which appears to be destined for the cell interior. The late population may be re-routed by chloroquine as proposed above.

There is an important aspect to chloroquine's effects which only serves to further support the proposition of Tf uptake mediation by pinocytosis, that is, its ability to depress the return of receptors to a cells surface. Such receptors appear to be held in an internal pool (Ciechanover et al., 1983; Schwartz et al., 1984; Tolleshaug and Berg, 1979 and; Zijderhan-Bleekemolen et al., 1987). Inspite of diminished receptor numbers, as well as the great amount of competing diferric human Tf, rat Tf is uptaken in the presence of chloroquine comparably to when it isn't present (see Figure 9.6).

A final issue to be discussed here concerns the divergence of Tf protein accumulation and iron accumulation observed in the presence of chloroquine (see Figure 9.8). This observation may be explained in the following way. Chloroquine is known to be a lysosomotropic agent capable of raising the pH of lysosomes and endosomes (Maxfield, 1984). Iron release from Tf is believed to require a decrease in pH in the surrounding milieu (Young and Bomford, 1984). It is reasonable therefore to assume that the compartment(s) wherein Tf releases its iron is(are) affected by the increase in pH produced by chloroquine, and thereby decreases the ability of iron to be released. Concerning this point, it is interesting to note the relatively limited reduction in iron uptake (Figure 9.7) and accumulation (Figure 9.8) which occur in the presence of the chloroquine. If, indeed as current dogma suggests, iron is released from Tf predominantly through a reduction in the local pH then it is troublesome to explain why chloroquine did not have a more profound effect. An alternative proposal has been advanced by Morley and Bezkorovainy (1985), Low et al. (1986 and 1987) Thorstensen and Romslo (1984a) and Cole and Glass (1983) wherein chemical reduction at the cell's surface releases iron from Tf to an awaiting acceptor molecule (Nunez and Glass, 1983).

If this is the mechanism by which iron is removed from Tf then obviously not all molecules taken up by fluid-phase endocytosis will be close enough to the cell surface to ensure release of all of the iron from all of the Tf (particularly if it is assumed that release of iron occurs at or very near the cell surface). As a result, less than 100% of iron bound to Tf taken into the cell would be released to the cell - a view supported by the present finding of radioiron release as illustrated in Figure 9.5. This implies that there must be release from the hepatocyte of some Tf molecules intact with respect to iron load or at most relieved of a single atom of iron. With respect to what ever role the decrease in pH may serve in this process, the latter suggestion appears most likely in the light of results in Chapter 4 concerned with ease of release of the N-terminal atom of iron.

In conclusion, mixed-type pinocytosis would seem to be the mechanism best able to explain the data presented and would therefore appear to be the premier candidate to mediate uptake of Tf by an adult rat hepatocyte.

CHAPTER 10

DIRECTIONS FOR FUTURE RELATED RESEARCH

10.1 Introduction

THE INTERACTION of rat transferrin with the liver is a complex event. Many interrelated variables such as iron co-ordination (Bates, 1982), the protein structure (Aldred et al., 1984), the site of iron release (Van Berkel et al., 1987), the glycan structure (Rudolph et al., 1986), and the pH environment of iron release (Lestas, 1976), are involved in the delivery of iron to cells. One of these in particular, the glycan structure on rat transferrin (RTf), has been shown here, for the first time, to play a modulatory role in this interaction. Furthermore, it has been demonstrated that iron delivery to hepatocytes does not proceed only by the transferrin receptor (TfR) but at least, adsorptive and fluid-phase pinocytosis are also involved.

In this study it has been demonstrated that rat transferrin contains a glycan of considerably greater heterogeneity than was previously appreciated (Schreiber et al., 1979; Gordon and Louis, 1963). Indeed, at least as many as six different subforms exist based on glycan microheterogeneity (Chapter 2). The subforms of

transferrin which are most prevalent in the plasma of the systemic circulation of a rat are the Con A distinguished subforms. It was these subforms that were studied here in detail. The purpose of this chapter is to discuss questions raised by results presented in this thesis and to suggest approaches for further study in order to answer those questions.

10.2 Diferric Rat Tf's Ability to Reduce the Enhanced Delivery of Iron to Hepatocytes by RAsTf.

Desialylation of the Con A subforms of rat transferrin (resulting in RAsTf) was found to result in dramatic changes in some of their functional properties (Chapter 3). In particular, their ability to donate iron to the liver was enhanced by 3-6-fold in comparison with their sialylated counterparts. Results from further experiments in vivo (Chapter 5) lead to the proposal that RAsTf interacts with the asialoglycoprotein receptor (ASGP-R or lectin) which, it was suggested, acts synergistically with the transferrin receptor (TfR) to allow for preferential uptake of RAsTf. Studies in vitro (Chapter 6) provided support for this proposal. An issue left unresolved from experiments in Chapter 6 (section 6.3.1) concerned the blocking effect of RTf on

RAsTf. Two related proposals were offered as possible mechanisms; RTf sterically hindered the RAsTf either: a) from interacting with the ASGP-R or b) from interacting with the cell surface once attached to the ASGP-R. An approach to resolve this question could be through examination of the ability of RTf to interfere with the binding of other asialoglycoproteins, such as asialofetuin, to the ASGP-R. Studies of the binding affinity of asialofetuin to freshly isolated hepatocytes or liver plasma membranes, in the presence of increasing amounts of diferric human or rat transferrin could potentially settle two issues: 1. Whether Tf causes steric hindrance of RAsTf interaction with the ASGP-R and; 2. Whether there is a difference in efficacy between RTf and HTf. If sialylated transferrin does interfere with the interaction of RAsTf with the ASGP-R, then it should be able to decrease the apparent association constant of asialofetuin. If there is no effect on the affinity of asialofetuin, then such a result would favour the interpretation that lectin binding is a first step of interaction with the hepatocyte followed by surface interaction and endocytosis, presumably steps that the ASGP-R enhances. If the transferrins do sterically hinder interaction with the ASGP-R, and if it is found that human transferrin more effectively alters affinity

than rat transferrin, then herein would be another explanation for the superior ability of human transferrin to block the enhanced iron donation to the liver in vivo. (In addition to the proposal of different local concentrations of transferrin in the space of Disse (see Chapter 6, section 6.4.1).)

10.3 Resolving Potential Functional Differences Between Sialylated Transferrins

Uncovering functional differences between the sialylated transferrins required the implementation of refined techniques, such as the paired protein studies of catabolic rates in the same animal as well as the use of isolated hepatocytes in suspension.

As stated in the introduction of Chapter 3, Gordon (1962) compared catabolic rates of individual proteins in separate animals. The studies here revealed that differences only become apparent by studying a pair of proteins in the same animal. Indeed, the ability of the different transferrins to donate iron to the liver should, ideally, be studied in this manner i.e. with one transferrin subform labelled with ^{55}Fe and another with ^{59}Fe. As well, as stated in Chapter 3, section 3.4.4, plasma disappearance of iron from transferrin should be studied using this approach.

Such experiments in the whole animal could reveal subtle differences between the subforms of transferrin, or they might not. However, a negative finding would not necessarily rule out the possibility of fundamental differences: The results obtained in this study from the liver in vivo compared with those from hepatocytes in vitro demonstrate this point. Results from studies in vivo constitute the sum of many interactions of transferrin with different sites in the body. The final result is analogous to examining a protein and asking for net ionic charges on the protein. The total protein has a single isoelectric point whereas at any one site the charge might be negative, positive or neutral depending on the relevant constituent amino acids under examination. The point here is, that it is important to appropriately address the issue. Therefore, experimental methods should be developed which can circumvent the obscuring effect the whole body studies can cause. The above mentioned paired studies in vivo is one such approach, but, at the same time the results could be augmented by comparatively investigating different sites of iron deposition throughout the animal, such as the bone marrow or the spleen, for their relative avidity in acquiring iron from the different subforms.

Another approach would be the use of cell suspensions or culture of cells from the different sites just mentioned or Sertoli cells (Skinner et al., 1984) as a further example. Finally, such experiments could be performed with animals (and/or cells) at different ages (Bolla and Greenblatt, 1982) or in different metabolic states such as iron deficiency or inflammation, in order to address the question of whether such states affect the differential interaction of the subforms of transferrin.

As mentioned above, the transferrin receptor does not account for all of the iron delivered to hepatocytes in vitro by transferrin. As inferred from results in Chapter 9, contributing mechanisms are probably adsorptive and fluid-phase pinocytosis. In the light of the fact that RTf-1 and RTf-3 subforms can be competed against with respect to iron delivery by increasing amounts of the respective subform, it seems reasonable to conclude that the glycan plays a modulatory role (as was suggested in the discussion of Chapter 3). A next possible step to help confirm or reject the hypothesis would be to conduct similar experiments with the respective aglyco-subforms.

10.4 Mechanisms Which Cause Microheterogeneity

Experiments presented in this thesis did not directly address the question of how glycan microheterogeneity of rat transferrin occurs, however it seems relevant at this point to discuss this issue and suggest how the question could be answered. As already discussed in Chapter 1, section 1.3.3, there are a number of ways in which different proteins can have different glycan structures. The truly intriguing question is how the same protein can end up with different glycans. Certainly, if the amino acid sequences of RTf-1, RTf-2 and RTf-3 turn out to be different, then in fact we would be discussing three different proteins. However, until evidence to the contrary is available, it is assumed here that the primary structure of all three subforms is the same.

It will be remembered that the decision to glycosylate or not occurs during protein synthesis or very shortly afterwards: within the first five minutes of synthesis, rat transferrin is glycosylated, takes on significant tertiary structure and before reaching the Golgi is found to contain all disulphide bonds (Morgan and Peters Jr., 1985). The glycan structure transferred to the nascent protein is the precursor glycan illustrated in Chapter 1, Figure 1.3(1). All glycoproteins begin with this structure and thus, it is beyond this

stage, in the Golgi, where the factors which cause microheterogeneity in rat transferrin must act. These factors are the make-up of the processing apparatus in the Golgi and the protein's intrinsic structural properties as brought about by its constituent amino acids, disulphide bonds, and position of the glycan. (This latter aspect with respect to position of the glycan was discussed more completely in Chapter 2.)

I. The Processing Apparatus in Golgi

With respect to the processing apparatus, the substrate specificity of the glycosyltransferases has been implicated as a major factor in controlling the synthesis of the glycan structure visa vis the so-called one linkage, one glycosyltransferase hypothesis (Schachter, 1984). Different cell types each contain a different processing apparatus and it is likely that the same polypeptide processed in different cells would result in different patterns of glycosylation (Yet et al., 1988). Indeed, experimental evidence supports this proposition (Noroika et al., 1985; Parekh et al., 1987). A variant of this proposition evolves from knowledge that a number of sites of transferrin synthesis exist within the body (Aldred et al., 1987). Possibly each site contains different processing machinery in their respective Golgi complexes and as a result

produce a transferrin with a different glycan structure. This latter hypothesis could be tested by culturing cells from different sites of production and examining the glycan structures on the synthesized transferrins, e.g. T-lymphocytes (Lum et al., 1986) or Sertoli cells (Skinner et al., 1984). In the same vein, it is also possible that separate populations of cells in the liver synthesize one or other form of transferrin and collectively the six (at least) different forms are produced. A way of testing this possibility is by creation of hybridomas with individual liver cells and examining the glycan on the transferrin produced. However, there is some evidence that cultured mouse hepatoma cells synthesize three different forms of transferrin based on electrophoretic analysis (Papaconstantinou et al., 1977) therefore it is possible that all liver parenchymal cells in the rat synthesize the different subforms of transferrin.

If each liver parenchymal cell is responsible for the production of all subforms of transferrin, then intracellular factors might alter the processing apparatus of the Golgi in such a manner as to result in the subtle microheterogeneity. Such factors could include iron, heme and possibly transferrin itself. Experimental manipulation of such factors in hepatocytes

in culture and examining the products could help decide this possibility.

II. The Protein Matrix

Yet et al. (1988) have demonstrated that the protein matrix itself can influence the appearance of the final glycan. The conformation of transferrin is known to be altered by its iron content wherein as many as four different conformations are possible (Makey and Seal, 1976). Morgan and Peters, Jr. (1985) have shown that newly synthesized rat transferrin is capable of binding iron before it reaches the Golgi. Thus, experimentally the influence of iron co-ordination on the final glycan structure could be tested as follows. First, in a limited fashion, by comparing the glycan on transferrin synthesized in iron deficient and iron replete animals. Second, and more effectively, by comparing the transferrin products from cultured cells maintained in a very low iron state by Desferal, with the transferrin products from control cultured cells.

Whether the ideas proposed here to elucidate the mechanism(s) of glycan production and functional significance in transferrin are completely or even partly valid remains to be tested by experimentation.

It is such experimentation that helps reveal the hidden aspects of this molecule, transferrin, which has been known to mankind for over forty years, but which continues to surprise and intrigue the imagination.

APPENDICES

APPENDIX CH-1

SUPPLEMENTARY NOTES TO CHAPTER 1

NOTES TO CHAPTER 1

1.1

More precise estimates had to wait a further 20 years: Work on the primary sequence of human transferrin by MacGillivray et al. (1983) indicated that there were 679 amino acid residues. Employing complementary DNA (Uzan et al., 1984) produced from the human transferrin gene, located on chromosome 3 (Young et al., 1984), gave results which translated into a complement of 678 amino acids for transferrin. This information coupled with the fact that each molecule of human transferrin contains two asparagine or N-linked glycans, which account for approximately 6% of the total weight (Baldwin and Egan, 1987; Aisen and Listowsky, 1980), has allowed the molecular weight to be established at 79 550 Da.

1.2

The amino- or N-terminal domain is composed of amino acids 1 through 336 and contains 8 disulphide bonds (see Figure 1.1) while the carboxy- or C-terminal domain is represented by amino acids 337 to 678. Within this latter domain there are 11 disulphide bonds. The two N-linked glycans mentioned above, are located at aspara-

gines 413 and 611 (MacGillivray et al., 1983) in the C-terminal domain and express considerable microheterogeneity (which will be discussed more completely below - see section 1.3.3). In addition, both domains of transferrin are very similar and are thus referred to as homologous domains (Figure 1.1). It had been suggested that the domains probably came into existence through gene duplication (Williams, 1982; Aisen and Listowsky, 1980) and a report by Park et al. (1985) confirmed this view. These workers isolated two overlapping human transferrin genomic clones from a liver DNA library and characterized them. It was stated that the duplication event probably occurred by unequal crossing-over and by recombination between sequences. Since the time of duplication the two domains have evolved somewhat independently (Williams, 1982). The success of the duplicated gene, and its product, a larger transferrin molecule (40 000 --- 80 000 Da) probably coincided with the advent of the filtering kidney system (Huebers and Finch, 1987).

1.3

Other chelated metals: copper (Zweier et al., 1979), gallium (Harris and Picoraro, 1983), chromium, cobalt, manganese (Aisen et al., 1969), vanadium (Harris

and Carrano, 1984), aluminum (Martin et al., 1987) and zinc (Evans, 1976). However the relevance of these *in vitro* findings with respect to the *in vivo* setting is not clear (Huebers and Finch, 1987).

1.4

The identity of the interacting sites in the transferrin molecule which act as ligands in the coordination of these iron atoms has been extensively investigated over the past 40 years. Only recently though, has it become reasonably clear which sites are most important.

1.5

The first observation which suggested that more than the protein was involved in co-ordinating iron binding was by Schade et al. (1949) wherein they reported the requirement of an anion, in particular bicarbonate, which was essential for the full development of the "salmon-pink colour" and that the stoichiometric relationship was one molecule of sodium bicarbonate per atom of iron. Work by other investigators demonstrated that probably bicarbonate or the carbonate anion was the physiological anion involved at one of the coordinating sites (Aisen and Leibman, 1973; Aisen et al., 1978;

Bates, 1982; Foljtar and Chasteen, 1982; Chasteen, 1983; Baldwin and Egan, 1987) however other anions (eg. malonate or oxalate) could substitute but with differing efficacy (Aisen and Leibman, 1973).

1.6

The second and only other non-transferrin participant in holding Fe^{3+} in position is a water or hydroxide molecule. The most convincing evidence of this was provided by the work of Koenig and Shillinger (1969a, 1969b) in which comparison of the solvent proton relaxation of apotransferrin and diferric transferrin yielded this view. It has recently been strengthened in extended-X-ray-absorption-fine-structure studies by Garratt et al. (1986) who came to the same conclusion.

1.7

A more recent report (Baldwin and Egan, 1987) suggests that the tyrosine at position 95 is more likely to be involved as opposed to Tyr 185 indicated in Figure 1.2. This latter configuration is depicted in three dimensions as the inset to Figure 1.2. Because of the high degree of homology, the binding sites in both C- and N-terminal domains are likely to be very nearly identical. The following table lists the relevant amino

acid residues, each of which is fully conserved between both domains and not only in human transferrin but also in hen ovotransferrin (Abola et al., 1982).

TABLE 1*
Some Functionally Important Amino Acid Residues

N-terminal Domain (1-336)	C-terminal Domain (337-678)
his(119); his(249); his(207)	his(451); his(585); his(535)
tyr(95); tyr(188); tyr(185)	tyr(426); tyr(517); tyr(514)
arg(124); arg(232); arg(254)	arg(456); arg(573); arg(590)

*After Baldwin and Egan, 1987.

1.8

However it is possible to achieve site-directed loading depending upon the form in which the iron is presented and the prevailing pH. The N-terminal domain is loaded first at neutral pH and in the presence of Fe^{3+} presented as a chelate of citrate or oxalate or as the salt of ammonium sulphate or chloride. While ferric iron chelated to nitrilotriacetate (NTA) is preferentially sequestered by the C-terminal site, or the so called A site (Aisen et al., 1978; Evans and Williams, 1978). In spite of the structural similarities between the two

sites, kinetic and thermodynamic differences exist (Baldwin and Egan, 1987).

1.9

It was first noticed by Surgenor et al. (1949) that transferrin begins to lose its specifically bound iron below pH 6.3. Lestas (1976) conducted equilibrium dialysis studies over a pH range of 4.2 to 7.5 and found that the N-terminal site is acid labile; the Fe^{3+} being released at a pH of approximately 5.7 - 6.0 while the second atom of iron is not released until pH 4.8. In a second type of study, Baldwin et al. (1982) used the chelator ethylenediaminetetraacetic acid (EDTA) over a pH range of 3.5 to 7.5 and found that the actual release of iron from the two sites probably involved different mechanisms. A direct attack by the chelate at the first or N-terminal site was suggested whereas the second-site requires a conformational change, which probably accompanies the decreasing pH, before the site directed attack by the chelator can occur (Baldwin et al., 1982; Bates, 1982; Cowart et al., 1986). A schematic representation of this chelate attack following a conformational change is presented in Figure CH-1.

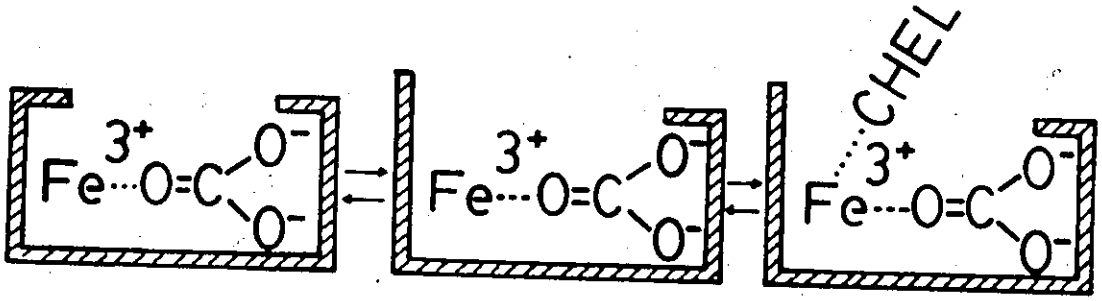


FIGURE CH-1

FIGURE CH-1 Schematic representation of Fe^{3+}-transferrin-CO_3^{2-} undergoing a rate limiting labilization of the Fe^{3+} and conformational change. This is followed by attack of a chelator (CHEL) and formation of a quaternary complex. (After Bates, 1982)

1.10

In their experiments they found that reticulocytes preferentially took up iron from a particular site which they arbitrarily called site A. They demonstrated this by first half-saturating (level A) with radioactive iron (^{59}Fe) the total iron-binding capacity (TIBC) of a volume of plasma which had been made iron-free. This was incubated with reticulocytes until half of the activity was removed (level B). A control sample of plasma was labelled with radioiron to the percentage saturation level B. Uptake of iron from both samples by reticulocytes was then measured. Radioiron uptake from the control sample was 61% greater than the pre-incubated plasma. It was suggested that some iron bound to transferrin was preferentially available to reticulocytes and that most was removed from the plasma sample which was pre-incubated. A control uptake experiment where radioiron was added to both samples at level B to return them to level A revealed similar uptake. To rule out the possibility of preferential uptake from saturated transferrin rather than from half-saturated transferrin, iron was added to both pre-incubated and control samples (at level B) to saturate TIBC. The 61% difference in uptake by reticulocytes persisted. "In these conditions only $Tf(Fe)_2$ molecules are present, and so the difference must be caused by different rates of uptake from some of the binding sites." (Fletcher and Huehns, 1967)

1.11

Support for the Fletcher-Huehns hypothesis (as it came to be known) was provided by a number of investigators including Awai et al. (1975) who conducted their experiments in vivo, asking whether doubly labelled transferrin selec-

tively deposited iron at various sites within the whole animal. In particular they examined the ratio of ^{55}Fe(A site)/^{59}Fe(B site) in the plasma, red blood cells (RBC), marrow heme, spleen heme, whole liver, Kupffer cells and parenchymal cells. They found, as did Fletcher and Huehns (1967), that the A site preferentially donated Fe to the RBC, marrow heme and spleen heme while the B site directed iron to the other tissues mentioned. Later experiments from the same group (Okada et al., 1978) further supported the hypothesis and also suggested that at low pH, even in the presence of citrate, there was no scrambling of iron between or within transferrins. However the major problem with these experiments related to the preparation of the double labelled transferrin. It was prepared exactly as had Fletcher and Huehns, i.e., incubation with reticulocytes and re-loading of iron depleted material with the second label. Pootrakul et al. (1977) in similar kinds of <u>in vivo</u> experiments also used this system of double-labelled transferrin but used different chelators and ammonium sulphate salts of iron to charge the transferrin. The above mentioned studies used NTA exclusively. These investigators found no significant difference from 1:1 in the ratio of $^{55}Fe/^{59}Fe$ distributed to the various tissues, except in 2 out of 5 experiments with NTA charged transferrin. They suggested that NTA's preferential distribution of Fe to the A-site

may have influenced their results and Awai et al.'s (1975) results. Further, and more importantly, they found as did Awai et al. (1975) that ^{59}Fe was favourably deposited to the liver but they concluded that this was probably due to contamination from ^{59}Fe hemoglobin (Hb). The source of this Hb was broken cells during the incubation with reticulocytes. Unfortunately though, at no point and in no way was the distribution of this radioiron ever verified in any of these experiments. Makey and Seal (1976) developed a technique based on electrophoresing proteins through a urea polyacrylamide gel which allowed resolution of the four molecular forms of transferrin: apo transferrin, monoferric at the N-terminal, monoferric at the C-terminal and diferric. It was a powerful technique which finally allowed investigators to establish exactly what the distribution of iron was in their preparations of human transferrin. Van Baarlen et al. (1980) exploited the technique and demonstrated that predominantly the N-terminal site of rabbit transferrin delivered iron to rabbit reticulocytes whereas with human transferrin delivery was mediated by the C-terminal site. However, their results are not convincing. While they took care to rule out the possibility of chelate mediated redistribution of iron between sites, they did not use labelled transferrins. Significantly, as time progressed

in their experiments, both sites became equipotent donors in both species. The crux of their argument was supported by a combination of the urea-PAGE technique and visualization of the different iron containing transferrins by crossed immunoelectrophoresis. They stated that apo and diferric transferrin have different reactivities with anti-transferrin antibody but no mention was made of potential differences in reactivity between the monoferric transferrins. Further, while more data was obtained with regard to rabbit transferrin, a peak of activity which corresponded to the N-terminal transferrin as distinguished from the peak of activity corresponding to the C-terminal transferrin was never demonstrated. From personal observations, urea gel electrophoresis followed by immunoelectrophoresis is incapable of resolving the N- and C-terminal forms of monoferric rat transferrin. It is possible that this is also true for rabbit transferrin and therefore would not allow Van Baarlen et al. to draw conclusions with respect to distribution of iron between sites.

It appeared as though the death knoll was sounded for the Fletcher-Huehns hypothesis in two sets of experiments by Huebers et al. (1978) and Van der Heul et al. (1981). The latter group incubated bone marrow cells with the different monoferric transferrins, which were

selectively labelled at differing pH and purified by preparative isoelectric focusing in granulated gels. It was found that the uptake of iron, in both a homologous (human) and heterologous (human transferrins; rat cells) system, was indistinguishable between transferrins. In the study by the first group, rat transferrin was used and care was taken to distinguish between the isotransferrins. Again, isolation of these monoferric transferrins was by isoelectric focusing however reticulocytes were used in the incubation. These investigators also found no difference in rates of iron donation between monoferric transferrins or between isoforms of rat transferrin.

The use of isoelectric focusing based on ampholites between pH 5-7, to isolate monoferric transferrins has been criticized (Leibman and Aisen, 1979; Zak and Aisen, 1986); however both this technique and the combined urea gel and crossed immunoelectrophoresis technique have been able to demonstrate similar results concerning monoferric transferrins. In particular it has been demonstrated that there is non-random distribution of iron to the A and B sites of transferrin in vivo by Leibman and Aisen (1979) and Zak and Aisen (1986) who used the combined technique while Van Eijk and Van Noort (1986) used the isoelectric focusing.

These three groups suggest that the B site is normally more fully occupied. This finding was also confirmed by an alternative approach wherein radioiron was administered to rabbits by epigastric tube (Marx et al., 1982). The iron was subsequently found to be predominantly bound to the N-terminal site of rabbit transferrin. These studies are at variance with a number of studies based on isoelectric focusing by Huebers et al. (1981, 1984) who suggest that the distribution of iron between the sites is random. Clearly a final answer to this important unresolved issue of random versus non-random distribution of iron between sites would help to finally establish the validity of the Fletcher-Huehns hypothesis.

At this point however, it seems reasonable to ask, even if there is a difference in delivery between sites, what impact would this have on the living organism if the bottom line is simply adequate versus inadequate supply of iron?

1.12

Transferrin has been localized, by immunochemistry, to the oligodendrocytes in both gray and white matter of the cerebral cortex (Connor and Fine, 1986). While transferrin mRNA levels have been reported as being

highest in the choroid plexus of the lateral and third ventricles, it has also been found to be present in the medulla, thalamus, choroid plexus of the 4th ventricle, hypothalamus, cerebellum and pituitary (Aldred et al., 1987).

1.13

The best characterized alternative site of transferrin synthesis is the Sertoli cells of the testis. Both rat testicular and serum transferrin appear to have similar, if not identical amino acid composition. However, testicular transferrin has been demonstrated to contain almost twice as much galactose and approximately 20% more N-acetylglucosamine (GlcNAc) (Skinner et al., 1984), the significance of which is not yet clear.

1.14

Aldred et al. (1987) have found that the size of the mRNA in these three sites (liver, brain and testis) is the same, which agrees with the findings of Skinner et al. (1984) for testicular transferrin. These data were determined by analysis of tryptic peptide maps or reverse phase HPLC.

1.15

The gastric mucosal cells are believed to be the site of synthesis of gastric transferrin which is found to contain 40% more carbohydrate than serum transferrin (Baldwin et al., 1986). Interestingly enough, gastrin appears to be able to bind to gastric transferrin and because gastrin is able to bind trivalent metal ions, it may have a role in donating ferric iron from the diet to transferrin. (The role of transferrin in iron absorption is discussed further in section 1.5)

1.16

Jordan and Morgan (1970) have demonstrated that transferrin and not lactoferrin synthesis occurs in the mammary glands of rabbits and more recently Bradshaw et al. (1985) have shown that mammary gland explants from rabbits synthesize transferrin. Lee et al. (1987) demonstrated that the mammary epithelial cells from mouse synthesized an iron-binding protein which they concluded to be transferrin on the basis of specific immuno-precipitation, 2-D electrophoresis of V-8 protease digests and by Ouchterlony diffusion. They also suggested, as did Jordan and Morgan (1970) for rabbits, that in mouse milk the major iron binding protein is transferrin. That mammary tissue synthesizes transferrin

is clear, however the purpose of this transferrin is not. Certainly it does not appear to be present as a protein supplement to the suckling young since milk transferrin is not absorbed at this stage of development of the alimentary canal, however, the transferrin may act as a bacteriostat. As observed by Jordan and Morgan (1970) transferrin concentration appears to be high in animals that suckle their young only once a day. This long period between feedings would, if not for transferrin's iron sequestering ability, provide an excellent opportunity for the growth of bacteria.

1.17

On the other side of the cycle, this means that approximately 1 g of transferrin is catabolized per day in man and over almost a 3 000-fold range in body weights (27 g - 80 kg), it is found that mammalian transferrin turnover is proportional to the body weight to a power of 0.681 (Regoeczi and Hatton, 1980).

1.18

Adrian et al. (1986) who succeeded in isolating a clone of the 5' region of the human transferrin gene have been able to demonstrate that within the 5' flanking region and intron I, there are conserved sequences which are identical with or homologous to the regulating

elements responding to heavy metals, the glucocorticoid receptor and a putative acute phase reactive signal. These findings could help to explain the 2.4 fold increase in production of liver transferrin mRNA which is found to occur during iron deficiency (Idzerda et al., 1986). Corresponding increases were not found in the brain or testis. Idzerda et al. also found a doubling of transferrin protein in the gut. They, however, attributed this increase entirely to increased transport of transferrin in the bile to the gut. Considering the reports of transferrin synthesis in the stomach it would have been interesting had they examined the mRNA at this site.

1.19

This glycosylated molecule ($Man_{9(8)}GlcNAc_2$-N-transferrin) is translocated to the Golgi complex where, in the case of transferrin, trimming of the glycan by 1,2 mannosidases IA and IB as well as mannosidase II (Tulsiani et al., 1982) in coordination with GlcNAc transferase I (Hughes et al., 1983) occurs and starts the nascent glycoprotein on its pathway for further processing and evolution of its complex glycan structure. (Glycoproteins which receive only limited or no clipping

by the mannosidases remain as high mannose structures (Kornfeld, 1983).)

Factors which have been suggested to bear importance on the final complex glycan structure include: 1. The substrate specificity of the glycosyl-transferases which attach their particular substrates (nucleotide-sugars) to the acceptor substrate (saccharides already part of the glycan) (Beyer et al., 1981); 2. The distribution and density of transferases within the Golgi apparatus. Schachter (1974) postulated that glycosyl transferases were probably serially located within the Golgi i.e. initiating transferases would be expected in the Cis region while terminating transferases should be in the trans-cisternae, an idea supported by the experimental evidence of Goldberg and Kornfeld (1983); and 3. Transferase density or frequency of expression (Meager et al., 1975, 1976).

1.20

In particular, Ekbom et al. (1983) demonstrated that the differentiation of metanephric mesenchyme proceeds only if transferrin is present in the medium; the differentiation and appearance of full function in colon epithelial cells was also found to be dependent on transferrin (Chopra et al., 1987). Granulocyte pre-

cursors in vitro were found to be induced to the matured neutrophil granulocyte stage (Evans et al., 1986) and chick embryo neuronal cultures required transferrin (and insulin) in serum free media to support growth (Aizenman et al., 1986). Blastemas, which are important in regeneration of newt forelimbs, required the addition of transferrin in order to grow and differentiate, however in this study, the authors attempted to replace transferrin's activity with iron salts. They observed a positive effect but suggested that the transferrin in their media picked up the iron and delivered it to the cells. The problem with this work was that there was no attempt to strictly define the fate of the iron. Yet, it attempted to dissect the matter of whether transferrin itself is important for growth or the iron it carries. Further work in this line includes two groups who simultaneously reported that transferrin plays a critical role in the differentiation of erythroid cells. The first group (Shannon et al., 1986) used monoclonal antibodies against transferrin. Their cultures of human erythroid bursts were no longer able to differentiate and ultimately the cells died in the presence of the antibodies. The second group (Schmidt et al., 1986) in a similar kind of study employed a monoclonal antibody which prevented the recycling of transferrin to

the cell surface but did not affect its internalization. They found that cells which had been committed to differentiation were arrested by the monoclonal. In both investigations however, restoration of high intracellular iron levels mediated by iron-chelate complexes were able to neutralize the effects of the antibodies. Hagiwara et al. (1987) cultured myogenic cells from 11-day chick embryo in the presence of transferrin or an equivalent amount of iron as the Fe-citrate complex. In both cases the degree of differentiation achieved by the cells was identical which clearly implies that transferrin does not play a primary role in cellular differentiation. Although, in a study of the proliferation of lymphocytes by Brock et al.(1986) it was indicated that transferrin itself had to donate the iron because a variety of iron containing compounds, including Fe-NTA were unable to replace transferrin and cause proliferation.

1.21

Iron Absorption

Iron in the diet is available for absorption in a variety of forms. It may be presented to the gut as ferric or ferrous salts or as iron in iron-containing complexes; predominantly heme and ferritin. It appears as though all non-heme or ferritin iron atoms move into a

"common pool" of iron before absorption wherein ligands for iron, contained in the diet, form absorbable (e.g., with ascorbic acid) or non-absorbable (e.g., tannic acid) complexes (Hallberg, 1981). Iron is not freed from the porphyrin ring of heme but the whole complex is well absorbed (Derman et al., 1982). On the other hand, ferritin is not well absorbed unless the diet is supplemented with "enhancers" of iron absorption such as ascorbate (Derman et al., 1982). Within this scenario of iron absorption arose the question of whether transferrin mediates any iron uptake.

In 1976 Huebers et al. isolated two isotransferrins from rat intestinal mucosa. They suggested that these two transferrins existed in addition to the two isotransferrins of plasma (Gordon and Louis, 1963). Analysis of these transferrins suggested that one had more aspartate, asparagine and proline than serum transferrin while the other mucosal transferrin was found to contain less tyrosine and phenylalanine. The isoelectric points also differed from each other and the serum transferrins. Unfortunately there was no carbohydrate analysis performed. These results align well, in principle, with the later findings of Baldwin et al. (1986) that a gastric transferrin exists and that mRNA is identifiable in stomach tissue (Aldred et al., 1987).

The Huebers group switched their tact somewhat when they demonstrated that transferrin is excreted with the bile, indeed they suggested that plasma transferrin, when exposed to bile acids undergoes a shift in isoelectric points (Idzerda et al., 1986) which obviously fits with their earlier findings. (One wonders if their serum transferrin also loses and/or somehow gains amino acids along the way through the bile! - how else can their earlier finding of a "gut transferrin" with differing amino acid composition be explained?)

The question of the mode of iron absorption has been addressed in many studies which have been performed with isolated and closed, tied-off gut segments from normal and iron deficient animals. Huebers et al. (1983) demonstrated that no iron from transferrin was absorbed in the ileum, only in the duodenum and jejunum. They found that the rate of iron uptake was similar to that observed with ferrous salts bound to albumin but intimated that transferrin was the likely carrier because apo and diferric transferrins were able to resist the proteolytic damage imposed by the lumen of the gut whereas albumin was broken down. Furthermore, they stated that absorption from the jejunum occured as a function of dose and exhibited saturation kinetics. This latter point is in direct contradiction with the findings

of Schumann et al. (1986) who suggested that iron uptake is nonsaturable. Savin and Cook (1980) conducted a very careful study of iron transport from closed intestinal loops. They went to great lengths to demonstrate that the transferrin which they observed in the mucosa was not due to contaminating plasma (something which was usually not rigorously ruled out in other studies). They found that the mucosal transferrin varied with total mucosal uptake of iron and the proportion that was transferred to the body. Idzerda et al. (1986) also demonstrated that in iron deficiency, the concentration of transferrin protein in the gut is doubled. It is interesting that a 2.4-fold increase in mRNA for transferrin in the liver also accompanies this doubling of gut transferrin.

The most convincing studies which suggest that transferrin is not a key player in iron absorption have also been conducted in isolated gut segments. Simpson et al. (1986) examined whether human transferrin, mouse transferrin or human lactoferrin when charged with radioiron were able to participate in iron absorption in mouse duodenum. Measured uptake of radioiron was found to be lower than ^{59}Fe-NTA presented by itself or if ^{59}Fe was bound to bovine serum albumin. In a similar

experiment, the apo forms of the proteins were added individually with ^{59}Fe complexed to NTA. There was a highly significant decrease in uptake when compared to uptake by ^{59}Fe-NTA alone. These authors commented that given the concentration of transferrin they found in the gut, and assuming first order kinetics, that iron uptake by transferrin, for the growing mouse, is inadequate by an order of magnitude. Only if transferrin were present in the gut at a concentration of 3 mg/ml (intestinal fluid) might it be adequate. Even the highest estimates of transferrin concentration in the gut are far below this level. Schumann et al. (1986) have also found transferrin in the bile, but their results demonstrated that there was no stoichiometric relationship between mucosal transferrin and the iron ions transferred across the mucosal epithelium by rat jejunal and ileal segments. In another kind of study, Bezwoda et al. (1986) investigated the ability of achlorohydric human subjects to absorb iron from ^{59}Fe charged transferrin which was given by gastric tube. They concluded that while limited absorption of iron did take place, transferrin-mediated uptake probably did not play a significant physiological role. However, they concede it is possible that intestinal transferrin may behave differently.

Clearly, this issue of transferrin's role in iron absorption is not resolved. If it is an important contributor then it may be, in part, responsible for iron regulation with respect to total body iron balance. On the other hand, the intracellular pool of iron may play a more important role in this regard (Jacobs, 1980; Refsum and Schreiner, 1984).

1.22

Iron which is stored in ferritin finds itself surrounded by a molecule which consists of approximately 24 structurally similar subunits. The structural arrangement is a 4,3,2 symmetry which relates to the four-, three-, and two-fold axes of symmetry. Each subunit consists of 5 helices and a long L loop, structural features which are shared by other iron containing proteins such as cytochromes c and b_{562}. The other major deposit of iron inside a cell is a compound known as hemosiderin. It is believed to arise through proteolytic degradation of the ferritin protein shell within the lysosomal compartment (Crichton, 1984; Bomford and Monro, 1985). The iron which is exposed by cytosolic proteolysis is probably reduced by flavin mononucleotides and re-enters the ferrous cytosolic pool of iron. Cytoplasmic ferritin messenger translation is

thought to be controlled by entry of iron into the cell; in this way, new apoferritin subunits are created independent of new message from the nucleus (Bomford and Monro, 1985).

1.23

This transmembrane portion has been demonstrated to be important in respect of its hydrophobicity although the particular arrangement of the amino acids does not seem to matter; the receptor appears to be unaffected by the amino acid sequence (Zerial et al., 1987). The same investigators have (Zerial et al., 1986) found that this transmembrane segment functions as a signal peptide for the receptor.

1.24

The demonstration of the inhibitory effects of Desferal (an iron-specific chelator) on the growth and cell cycle of K562 cells (Bomford et al., 1986) supports this view. Coincident with this decrease in growth is a reduction in the expression of transferrin receptors. Terminal differentiation of cells also results in a decrease in transferrin receptor expression as demonstrated in K562 cells (Hunt et al., 1984), HL-60 cells (Ho et al., 1986), erythroid cell development (Iacopetta and Morgan, 1983b) and is implied by the difference in

receptor numbers on fetal hepatocytes (Trinder et al., 1986) compared with adult hepatocytes (Young and Aisen, 1980). On the other hand, factors which stimulate growth appear to exert a positive effect on receptor expression. Wiley and Kaplan (1984) observed a striking, but transient (5 min) shift of transferrin receptors from an internal pool to the cell surface of fibroblasts which was caused by epidermal growth factor (EGF). Davis and Czech (1986) made similar observations with EGF but in addition showed that insulin-like growth factors and platelet-derived growth factor resulted in effects for as long as 2 h. As well, Davis et al. (1986) observed a 3-fold increase in surface receptors on fat cells after stimulation by insulin. Finally, it has been shown that partial hepatectomy is capable of inducing an increase in transferrin receptors on hepatocytes 18 (Hirose-Kumagai et al., 1984) to 24 (Tei et al., 1984) hours after the surgery. However, which factors in particular are responsible for the effect during the regeneration of liver tissue, is not clear.

1.25

May et al. (1986) demonstrated a down regulation in HL-60 cells by a synthetic diacylglycerol capable of activating protein kinase C. As well, Hunt and Marshall-Carlson (1986) found the trifluoroperazine, which

enhances the phosphorylation of transferrin receptors, caused a reduction by one half of the receptors on the surface of K562 cells.

1.26

Louache et al. (1984) grew K562, HEL, HL-60 and U937 cells in the presence of ferric ammonium citrate or hemin and found a time-dependent decrease in the synthesis and expression of receptors. They suggested that hemin, protoporphyrin IX or iron may be the main regulators of transferrin receptor synthesis. A study by Rhyner et al. (1985) in HL-60 cells, have included intracellular ferritin on this list. In line with these findings is the work of Cox et al. (1985) who found that artificial depression of intracellular heme with cyclohexamide resulted in a significant decrease in receptor synthesis. In the same line of thinking but from the opposite perspective, Mattia et al. (1984) induced iron deficiency in K562 cells with Desferal and observed enhanced transcription of the receptor mRNA and increased expression on the cell surface.

APPENDIX CH-3

ISOLATION OF HEPATOCYTES

ISOLATION OF HEPATOCYTES

MATERIALS

Collagenase Type IV - Sigma

Purchase with collagenase activity as high as possible (minimum of 300 units/mg); clostripain as low as possible (maximum - 0.20 units/mg); caseinase less than 200 but greater than 100 units per mg; and tryptic activity as low as possible (maximum - 0.05 units/mg).

Hank's Balanced Salt Solution - HBSS (concentrated 10x for storage)

KCl	-	4.0g
KH_2PO_4	-	0.6g
NaCl	-	80.0g
$Na_2HPO_4.7H_2O$	-	0.9g
Glucose	-	10.0g
Phenol red		0.1g

all per litre.

1 L of this concentrated 10x HBSS should be stored in 10, 100 ml volumes. For use, dilute the 100 ml volume 1:10, as follows:

1. Make up to 1 L with distilled water.
2. Reserve 200 ml for step 8.

3. Add 8 mg Desferal and 1.68 g sodium bicarbonate to the remaining 800 ml. Use 600 ml for step 4 and 200 ml for step 5.
4. Pass the 600 ml volume through a 0.45 um Nalgene millipore filter, this is the INITIAL PERFUSATE. Place into a 600 ml beaker.
5. To the 200 ml volume which is the balance of the 800 ml, add 200 mg of BSA and 67 mg of $CaCl_2$. Divide this volume into two 100 ml volumes.
6. Pass one of the 100 ml volumes through a 0.45 um Nalgene millipore filter , this is the STIR SOLUTION. Place into a 250 ml Erlenmeyer flask.
7. To the second 100 ml volume, just before use, add 70 mg of collagenase. Pass through a 0.45 um Nalgene millipore filter. Place into a container equivalent to the bottom third of a 250 ml beaker (80 x 40 mm Pyrex dish). This is the COLLAGENASE SOLUTION.
8. To the reserved 200 ml, add 67 mg $CaCl_2$, 656 mg HEPES, and 2 g BSA. Adjust pH to 7.4. Pass through 0.45 um Nalgene millipore filter. This is the RESUSPENSION BUFFER.

The COLLAGENASE SOLUTION, 80 ml of the STIR SOLUTION and the INITIAL PERFUSATE are all warmed to 37°C. The RESUSPENSION BUFFER and the remaining STIR SOLUTION are reserved on ice.

ISOLATION OF HEPATOCYTES

1. The collagenase solution, the initial perfusate and the stir solution are all aerated with 95% O_2, 5% CO_2 (carbogen) while being warmed. At this point, 1,000 units of heparin are added to the initial perfusate.
2. The animal is placed into a plastic container, which is open only to a piece of tubing which carries the anesthetic (Halothane/N_2O/O_2 - generated by evaporating 23 drops/min of Halothane in a 150 ml Erlenmeyer flask contained in a water bath of 80-90°C and subject to 50 mm/Hg, N_2O and 30 mm/Hg, O_2.)
3. The rat is subjected to this anesthetic in the container for five minutes, then removed and placed ventral side up and anesthetic continued. At this point 1,000 units of heparin are injected i.p.
4. When the proper level of anesthesia is achieved (no response to pinching) coat the abdomen with 70% ethanol.
5. Expose the abdominal viscera. Move the intestines to the animal's right side, revealing the portal vein.
6. Place two ligatures (4-0 silk) around the vein, one proximal to the liver and one distal. (Approximately 5 mm apart.)

7. Tie a very light knot on each ligature - to be tightened once the cannula is in place.
8. Ensure that the cannula (PE-50) and its connected silastic tubing are free of air bubbles. This line runs from the initial perfusate through a pump, into an air trap then to the cannulating tip.
9. Adjust the flow rate of perfusate to 20 ml/min then turn off the pump.
10. Cannulate the vein, secure the first ligature with one tie then turn the pump on. This should be accomplished within 2 min. Increase the flow rate to 30 ml/min then cut the inferior vena cava. Secure both ligatures. Pinch off the cava to "inflate" the liver. If the liver is completely "blanched" proceed, if not, it is not worth continuing, start with a new liver.
11. Excise the liver (approximately 10 min), then carefully suspend in the collagenase solution. Switch perfusion solutions correspondingly (mixture of solutions should be held to a minimum). Perfuse for 10 min. At 5 min increase the perfusion rate by 2 ml/min. Increase it to the same extent at 7 min and 9 min.
12. Stop perfusion at 10 min. Remove the liver, gently. Place it onto a watch glass on ice. Cut away the

capsule around each lobe. With a Pasteur pipette, "spray" the 20 ml of cold STIR SOLUTION onto the liver. This procedure should dislodge most of the cells. Free remaining attached cells by gently shaking the liver in the solution. BE PATIENT and GENTLE.

13. The suspension is then transferred to the warm 80 ml of STIR solution and the Erlenmeyer swirled at 100 rev/min at 37°C with aeration with carbogen. After 30 min. the initial suspension is filtered through a stainless steel mesh and then the cells allowed to sediment by gravity for 20 min. on ice.
14. The supernatant is then removed, and cells resuspended to 40 ml with cold RESUSPENSION BUFFER. The cells are then centrifuged at 40 g for 30 s. The supernatant is discarded and cells resuspended to 40 ml then centrifuged at 40 g for 30 s. This is repeated twice.
15. Viability, cell numbers and contamination with non-parenchymal cells are then determined by trypan blue exclusion using a hemocytometer and a microscope.

APPENDIX CH-6

ANOVA TABLES

APPENDIX CH-6

ANOVA TABLES FOR DATA IN FIGURES 6.1, 6.2, 6.3, AND 6.5

FIGURE 6.1A

SOURCE	DF	SS	MS	F	
Total	19	66.5	-		
Proteins	1	4.53	4.53	7.19	p<0.00001
Time	4	53.15	13.29	21.09	
Error	14	8.82	0.63		

FIGURE 6.1D

SOURCE	DF	SS	MS	F	
Total	19	1.14	-		
Proteins	1	0.0002	0.0002	0.01	p= 0.92
Time	4	0.809	0.202	9.97	
Error	14	0.304	0.0217		

FIGURE 6.2B

SOURCE	DF	SS	MS	F	
Total	19	0.23	-		
Proteins	1	0.016	0.016	1.03	p= 0.327
Time	4	0.597	0.149		
Error	14	0.217	0.0155		

FIGURE 6.2C

SOURCE	DF	SS	MS	F	
Total	19	21.73	-		
Proteins	1	0.01	0.01	0.138	p= 0.713
Time	4	20.706	5.176		
Error	14	1.014	0.0724		

FIGURE 6.2D

SOURCE	DF	SS	MS	F	
Total	19	80.98	-		
Proteins	1	1.05	1.05	23.03	p= 0.0003
Time	4	79.291	19.323		
Error	14	0.639	0.0456		

FIGURE 6.2E

SOURCE	DF	SS	MS	F	
Total	19	7.665	-	-	
Proteins	1	0.640	0.640	4.809	p= 0.0457
Time	4	5.162	1.290		
Error	14	1.863	0.1330		

FIGURE 6.2F

SOURCE	DF	SS	MS	F	
Total	19	78.77			
Proteins	1	0.01	0.01	0.06	p= 0.810
Time	4	76.454	19.114		
Error	14	2.306	0.164		

FIGURE 6.3A

SOURCE	DF	SS	MS	F	P
Total	59	8.521			
Time	5	7.0380	1.4076	57.05	<0.0009
Groups	4	0.377	-	-	
Proteins	1	0.126	0.126	5.106	0.0279
Exp.	3	0.251	0.083	3.809	0.0084
Error	55	1.357	0.0246		

FIGURE 6.3B

SOURCE	DF	SS	MS	F	P
Total	47	5.365	-	-	-
Time	5	4.4197	0.8839	47.39	
Groups	3	0.218	0.073	3.91	0.0156
Proteins	1	0.005	0.005	0.268	0.607
Error	39	0.7273	0.01864		

FIGURE 6.3C

SOURCE	DF	SS	MS	F	P
Total	47	10.643	-	-	-
Time	5	7.0335	1.4067	22.65	
Groups	3	1.188	0.396	6.377	0.0013
Proteins	1	0.285	0.285	4.59	0.0385
Error	39	2.4215	0.0620		

FIGURE 6.5A (No Excess RTf)

SOURCE	DF	SS	MS	F	
Total	23	3.487			
Proteins	1	0.005	0.005	0.165	p=0.6897
Time	5	2.9694	0.5939	19.69	
Error	17	0.5126	0.0030		

FIGURE 6.5B (No Excess RTf)

SOURCE	DF	SS	MS	F	
Total	23	0.3077			
Proteins	1	0.0046	0.0046	1.151	p=0.298
Time	5	0.23517	0.04703	11.769	
Error	17	0.06793	0.00399		

DF, degrees of freedom; SS, sum of squares; MS, mean square; F, ratio of mean square of source to mean square of error.

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