The Biosynthesis of Rat Transferrin
EVIDENCE FOR RAPID GLYCOSYLATION, DISULFIDE BOND FORMATION, AND TERTIARY FOLDING*

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The transit time of newly synthesized transferrin in the liver is markedly longer than that of albumin. We sought to learn the basis of this difference by the use of labeled leucine and mannose in vivo and by isolation of newly formed transferrin from rough microsomes of rat liver. Albumin and α₁-antitrypsin, a second glycoprotein, were also studied for comparison. Minimal hepatic transit times were 17, 23, and 31 min for albumin, α₁-antitrypsin, and transferrin, respectively. The delay in the case of transferrin was found to occur chiefly in the rough endoplasmic reticulum and to be paralleled by an increase in the amount of transferrin relative to albumin in that organelle. Initial glycosylation of transferrin was as rapid as that of α₁-antitrypsin, and essentially all of the transferrin in the rough endoplasmic reticulum contained glycans which bound to concanavalin A and were removed by endoglycosidase H. Only 3% of the transferrin isolated from the rough microsomes came from the plasma by endocytosis or adsorption. Rapidity of disulfide bond formation in rough microsomes was evident from the presence of only 1.3 cysteine thiols/molecule of rough microsomal transferrin (total of 19 cystines) and the absence of mixed disulfides. Peptide patterns upon mild proteolysis were consistent with a native configuration of disulfide bond pairing. The ability of rough microsomal transferrin to bind and deliver iron through interaction with transferrin receptors on reticulocytes suggests that considerable tertiary structure is present. Thus, initial glycosylation, disulfide bridging, and tertiary folding are all rapid processes. The cause for the slow release of transferrin from the rough endoplasmic reticulum may lie with a rate-limiting transfer mechanism.

Transferrin and albumin are both synthesized by the liver, but with marked differences in their secretion kinetics (1–3). After the intravenous injection of [14C]leucine in the rat, the label is rapidly incorporated into both transferrin and albumin in the total microsomal fraction of the liver, but the labeled transferrin is much slower to leave this fraction and appear in the circulating blood than is the labeled albumin (1). Recent evidence of a semiquantitative nature indicates that the slower secretion of transferrin in vivo (4) or in isolated rat hepatocytes (5–7) is due to a delay in the passage of the protein from the endoplasmic reticulum to the Golgi complex. However, the cause of this delay is unknown. It is not due to the synthesis of the two proteins in different cells or to their passage through different intracellular organelles, since transferrin and albumin have been demonstrated immunohistochemically to occur in the same hepatocytes and in the same endoplasmic reticulum and Golgi vesicles of individual cells (8–10). Several other possible reasons are considered in this paper.

One possibility is that the delay in transferrin secretion results from the glycosylation process, transferrin being a glycoprotein while albumin is not. If so, other glycoproteins would be expected to show similar intracellular kinetics to transferrin. A second possibility is that the delay is due to the time involved in peptide chain folding and disulfide bond formation to give the transferrin molecule the tertiary structure which is required for its secretion from the cell. It is known that mammalian transferrin has a more complex tertiary structure and disulfide bond arrangement than does albumin (11, 12). A certain stage of either glycosylation or structural organization of the polypeptide chain may be required for interaction of the transferrin molecule with the secretory transport mechanism. Third, transferrin and albumin may compete for a common, limited-capacity transport mechanism, with the greater rate of synthesis and secretion of albumin resulting in a slowing of the secretion of transferrin. This possibility is suggested by the observation that transferrin secretion is accelerated in analbuminemic rats in comparison with normal rats (13).

The aim of the present study was to investigate the above problems in vivo in the rat. α₁-Antitrypsin, another plasma glycoprotein which is also synthesized in the liver (14), was included with transferrin and albumin in order to determine whether the delay in the secretory process is common to glycoproteins or is peculiar to transferrin. Experiments were performed to compare the rates of synthesis and secretion of the three proteins into the circulating blood, to determine the intracellular site of delay in the secretion of transferrin in vivo, and to see whether the amount of transferrin in the different intracellular fractions is in accord with its transport kinetics. Other studies were undertaken to test whether the delay in transferrin secretion is due to glycosylation, disulfide bond formation, or folding of the transferrin molecule or to its competition with albumin for a secretory transport mechanism. Since transferrin is known to be endocytosed and recycled in many types of cells, additional studies were per-
formed to determine what proportion of the transferrin isolated from the intracellular fractions could have arisen from the plasma.

**EXPERIMENTAL PROCEDURES AND RESULTS**

Incorporation of $^{3}$H]Leucine into Plasma Proteins—A different pattern of incorporation of $^{3}$H]leucine was observed with each of the three plasma proteins studied (Fig. 2A). Incorporation was most rapid with albumin and reached a plateau level sooner than with the other proteins. It was least rapid with transferrin and even after 180 min had not attained a plateau. For each protein, the time curve of secretion followed an exponential (Fig. 2B) with greatest slope for albumin ($t_m = 16$ min), intermediate slope for $\alpha_1$-antitrypsin ($t_m = 21$ min), and least slope for transferrin ($t_m = 28$ min). Extrapolation of these curves to zero incorporation measures the delay time between injection of the labeled leucine and its initial appearance in the secreted proteins. Values of 17, 23, and 31 min were obtained in this way for the minimal transit times of albumin, $\alpha_1$-antitrypsin, and transferrin, respectively, in good agreement with our earlier figures for albumin and transferrin of 16 and 31 min (1).

Incorporation of $^{3}$H]Leucine into Transferrin, Albumin, and $\alpha_1$-Antitrypsin in Intracellular Liver Fractions—The appearance of $^{3}$H]leucine in the proteins extracted from rough microsomes, smooth microsomes, and Golgi fraction is shown in Fig. 3. With all these proteins, peak values were reached in the above order in the three fractions. However, there were distinct differences between the results obtained with the three proteins, especially between transferrin and albumin. With all proteins, peak levels of $^{3}$H]leucine incorporation on the rough endoplasmic reticulum were observed at about the same time, 10–15 min, but the disappearance of radioactivity from this fraction was slowest in the case of transferrin and fastest with albumin. The peaks of appearance of labeled transferrin in smooth microsomes and Golgi fractions also occurred at later times (25 and 30 min, respectively) than with albumin (15 and 20 min) or $\alpha_1$-antitrypsin (about 17.5 and 25 min). Another difference was in the maximal level of radioactivity reached in the Golgi fraction. In the case of albumin the Golgi peak was approximately 90% as great as that in the rough microsomes, but with the other two proteins it was only about one-third of the value found in the rough microsomes.

Differences in the intracellular distribution of the proteins were also manifested by measurements of the concentrations of the proteins in the subcellular fractions (Table 1). Whereas similar concentrations of albumin were found in the rough microsomes and Golgi fractions, with transferrin and $\alpha_1$-antitrypsin, the Golgi levels were relatively lower, being approximately 25 and 50%, respectively, of those in the rough microsomes.

Incorporation of Mannose into Proteins of Intracellular Fractions—The incorporation of $^{3}$H]mannose and $[^{14}$C]leucine into transferrin, albumin, and $\alpha_1$-antitrypsin was followed for 30 min after their simultaneous intravenous injection. Fig. 4 illustrates the results obtained with the proteins extracted from the rough microsomes. The incorporation of $[^{14}$C]leucine was similar to that previously observed with $[^{3}$H]
TABLE I

Protein concentrations of liver subcellular fractions

| Protein | Rough microsomes | Smooth microsomes | Golgi fraction |
|---------|-----------------|-------------------|---------------|
| Total protein | 10,780 ± 462 | 2,710 ± 93 | 590 ± 36 |
| Transferrin | 73 ± 3.1 | 32 ± 1.2 | 19 ± 1.1 |
| Albumin | 61 ± 2.8 | 30 ± 1.5 | 65 ± 3.8 |
| α1-Antitrypsin | 16 ± 0.56 | 4.2 ± 0.44 | 8.2 ± 0.75 |

FIG. 4. Incorporation of [14C]leucine (⚫) and [3H]mannose (○) into transferrin, α1-antitrypsin, and albumin in rough microsomes. The basis is per weight of liver. Each value is the mean from two animals.

FIG. 5. Incorporation of [14C]leucine and [3H]mannose into transferrin and α1-antitrypsin in rough microsomes (⚫), smooth microsomes (○), and Golgi (▲) fractions. The basis is specific activity of the individual proteins. Each value is the mean from two animals.

leucine. No [3H]mannose was incorporated into albumin. With both transferrin and α1-antitrypsin, incorporation was low during the first 5 min and thereafter rose steadily until the end of the study at 30 min. Although the incorporation of [3H]mannose/unit quantity of transferrin was lower than with α1-antitrypsin (Fig. 5), there was no evidence of a delay in its incorporation into transferrin when compared with α1-antitrypsin.

The incorporation of [3H]mannose into transferrin and α1-antitrypsin in the smooth microsomes and Golgi fractions also showed a similar pattern for these proteins (Fig. 5). The values were higher for α1-antitrypsin, but with both proteins they rose steadily in each fraction during the 30 min of study and became progressively lower on moving from the rough to the smooth microsomes and then to the Golgi fraction.

Properties of Transferrin in the Rough Microsomes—We next sought to study the properties of transferrin as it exists in the rough microsomes to attempt to explain its prolonged retention in that organelle. Transferrin was isolated from Triton X-100/sodium deoxycholate extracts of rough microsomes by affinity chromatography with antitransferrin-agarose as described under “Experimental Procedures.” Yield was about 45 μg from rough microsomes of 1 g of liver. The extent of inclusion of transferrin from the circulation in the rough microsomal transferrin was tested by injecting [125I]-transferrin intravenously 3 h before removing the livers. Of the immunoprecipitable transferrin measured in the three cell fractions, only 4.5 ± 0.8% (S.E., n = 3), 7.1 ± 0.4%, and 3.7 ± 0.3% in rough microsomes, smooth microsomes, and the Golgi fraction, respectively, could be traced to transferrin adsorbed or taken up by endocytosis from the circulation. In the rough microsomal transferrin prepared by immunoprecipitation, only 1.3 ± 0.0% of a total of 43 μg/g, or 3.0%, was traceable to circulating transferrin.

Size and Composition—On electrophoresis in 7.5% polyacrylamide gels with sodium dodecyl sulfate and β-mercaptoethanol, rough microsomal transferrin showed a single band of the same molecular weight as transferrin isolated from serum. Judging from the migration of marker proteins, carbonic anhydrase (30,000), ovalbumin (43,000), bovine serum albumin (67,000), and phosphorylase b (94,000), the molecular mass of the mass transferins was 77,000 ± 500 daltons, close to the value of 76,500 reported for rat serum and total microsomal transferrin by Schreiber et al. (3). Amino acid composition of the rough microsomal transferrin (not shown) was found to be identical with that of serum transferrin within the precision of the analyses and to agree well with the composition of rat serum transferrin reported by Schreiber et al. (3).

Sialic Acid and Mannose Content—Upon electrophoresis on cellulose acetate at pH 8.7, transferrin from rough microsomes appeared as a single band with a slower anodic migration rate than that of serum transferrin, again in agreement with the work of Schreiber et al. (3) with transferrin isolated from the total microsomal fraction of rat liver. Treatment with neuraminidase did not affect the migration of rough microsomal transferrin, but slowed the migration of serum transferrin so that the two proteins moved at the same rate. This result suggests that the charge difference between the two transferrins is due to absence of sialic acid in the rough microsomal form. The transferrin prepared from rough microsomes, on the other hand, sensitive to Endo H.2 The rough microsomal transferrin, but not serum transferrin, showed an observable but slight decrease in molecular weight on polyacrylamide slab gel electrophoresis after Endo H treatment, similar to that reported by Nakada et al. (4) and Lodish et al. (6), and moreover a complete loss of staining for carbohydrate with concanavalin A plus peroxidase in Western blots. Hence, the major form of transferrin found in rough microsomes has properties similar to the high-mannose, Endo H-sensitive, uncharged species identified by the above authors using labeled mannose as ManαGlcNAcβ.

To test whether any unglycosylated transferrin could be detected in the rough microsomes, rats were injected with [3H]mannose and [14C]leucine, and at various times thereafter

The abbreviations used are: Endo H, endo-β-N-acetylglucosaminidase H; NaCl/Tris, phosphate-buffered saline (0.1 M NaCl, 0.016 M Na2HPO4, 0.0038 M NaH2PO4, pH 7.4); NaCl/Tris (0.1 M NaCl, 0.02 M Tris-Cl buffer, pH 7.4).
livers were fractionated with transferrin isolated by immunoaffinity from the rough microsomes. Binding of this transferrin to immobilized concanavalin A is shown in Table II. At each time point, virtually the same proportion of total $^3$H and $^{14}$C in the transferrins bound to the lectin. Although binding of [14C]leucine at the 5-min incorporation time was not quite complete, neither was the binding of [3H]mannose. This may be due to the presence of a small fraction of immature transferrin molecules in which the mannose units of the oligosaccharide chains are not available to complex with the lectin. In any event, there was no evidence for a transferrin form which contained labeled leucine but no labeled mannose.

Test for Unbonded Cysteines—Unbonded cysteines of rough microsomal transferrin were measured by the technique developed in an earlier study with serum albumin (22). Transferrin was isolated from rough microsomes following sudden perfusion of rat livers with 50 mM iodoacetamide to "freeze" unbonded cysteine thiols by alkylation. Analysis for carboxymethylcysteine showed $1.3 \pm 0.6$ (S.E.) mol/mol of rough microsomal transferrin ($n = 9$), compared to 0.0 mol/mol for serum transferrin also isolated by the immunoaffinity technique.

Test to Identify Mixed Disulfide Complexes—Mixed disulfide forms of transferrin of rough microsomes were sought by two methods. 1) No constituents other than the expected amino acids and amino sugars were detected with ninhydrin after acid hydrolysis, at a sensitivity of 1 mol/mol of transferrin. 2) Upon reduction of intact protein, no potential mixed disulfide constituents were observed at a sensitivity of 0.3 mol/mol of transferrin. Compounds which might have been observed included cysteine, glutathione, cytochrome c, and the mixed disulfide, cysteine-S-S-cystamine. Cystamine itself is too basic to be observed on the amino acid analyzer.

Disulfide Bonding Pattern—Whether disulfide bonds of rough microsomal transferrin were paired in their native configuration was tested by the peptide mapping technique of Cleveland et al. (23). Peptides were separated by gel electrophoresis following partial cleavage by a battery of proteases under nonreducing but denaturing conditions. Results are shown in Fig. 6a. Rough microsomal transferrin (Fig. 6a, lanes 1, 3, 5, and 7) showed patterns indistinguishable from those given by serum transferrin (lanes 2, 4, 6, and 8), also isolated by the immunoaffinity procedure, upon partial digestion with chymotrypsin, subtilisin, and papain at pH 6.5. Other enzymes tested, trypsin, pepsin, Staphylococcus aureus protease A, and pronase, did not give useful results, the digestion being either too limited or too extensive. To demonstrate that the technique is capable of detecting differences in disulfide bonding pattern, rough microsomal transferrin was also compared to partially alkylated serum transferrin (Fig. 6B). In this case, differences between the patterns of the two proteins are apparent with all three enzymes.

Iron- and Receptor-binding Properties of Rough Microsomal Transferrin—No significant differences were found between rough microsomal and plasma transferrins. The iron-binding capacity of the sample of rough microsomal transferrin was estimated to be 1.94 mol of Fe/mol of transferrin and of plasma transferrin 1.86 mol of Fe/mol of transferrin. The values for the affinity constants for the interaction with rat reticuloocyte receptors were 0.45 and 0.42 x 10$^{10}$ liter/mol for microsomal and plasma transferrins, respectively. At a transferrin concentration of 1.55 mM, the rates of iron uptake at 37 °C were 1.11 and 1.07 nmol ml -1 reticulocytes -1 min -1 for the microsomal and plasma samples.

Relative Retention of Transferrin and Albumin by Rough Microsomal Membranes—The release of H-transferrin from rough microsomes prepared 15 min after [3H]leucine injection was compared with release of [3H]albumin under various conditions. Only freezing and thawing caused a greater release of labeled transferrin than of albumin (16% compared to 9%). No differences were observed with sonication or 0.06-0.15% w/v, digitonin, 0.02-0.05% saponin, 0.05-0.1% Triton X-100, or 0.1 M Na$_2$CO$_3$ at 37 °C for 1 h.

**Table II**

Affinity chromatography of transferrin from rough microsomes on concanavalin A-agarose  

Rats were injected with [3H]mannose and [14C]leucine. Livers were removed at the indicated times, homogenized, and protein extracts prepared from the rough microsome fractions. Transferrin was isolated from the extracts with an immunoaffinity column and then applied to a column of concanavalin A-Sepharose 4B. The results show the per cent of total transferrin radioactivity which bound to this column and was subsequently eluted with 0.3 M $\alpha$-methyl-$\beta$-mannoside. [14C]Leucine-labeled albumin was not retained by the concanavalin A column. Each value is the mean result from two rats.

| Time (min) | Radioactivity bound by concanavalin A-agarose | % total radioactivity |
|-----------|-----------------------------------------------|----------------------|
|           | [3H]mannose | [14C]leucine       |                      |
| 5         | 88.8        | 89.8                |
| 10        | 96.8        | 97.0                |
| 15        | 97.3        | 96.9                |
| 20        | 95.8        | 97.0                |
| 30        | 98.5        | 98.0                |

**Discussion**

The measurement of [3H]leucine incorporation into the circulating proteins (Figs. 1 and 2) confirms and more precisely defines our earlier observations (1) of a greater delay and slower rate of secretion of newly synthesized transferrin than albumin. Similar differences between transferrin and albumin secretion have also been observed in the rat in vivo (3) and in isolated rat hepatocytes (7, 29) and rat hepatoma cells (5, 6, 30). The inclusion of $\alpha_1$-antitrypsin in the present study has shown that the secretion characteristics of this glycoprotein lie between those of albumin and transferrin, although they are closer to albumin than to the other glycoproteins, transferrin. Hence, the long delay and slow rate of secretion found with transferrin is not characteristic of all glycoproteins. On the basis of the results obtained with $\alpha_1$-antitrypsin, only a small proportion of the delay and slowing of secretion is attributable to the glycosylation process.

Secretion of the labeled proteins was not affected by the injection of cycloheximide 10 min after the [3H]leucine, in agreement with observations by Nakada et al. (4), even though the dose used was shown to inhibit plasma protein synthesis effectively. It is, therefore, apparent that the radioactive leucine was acting as a pulse with a duration of less than 10 min to label intracellular pools of the proteins from which they were subsequently secreted. The observation that the appearance of the labeled proteins in the plasma follows simple first-order kinetics (Fig. 2) implies that secretion occurs by random process from the intracellular pools. As pointed out previously (2), the label and first appearance of labeled protein in the blood stream indicates that a time-ordered mechanism is also involved in the secretory process. Both the delay times and the secretory kinetics varied between the three proteins (Fig. 2). Hence, each protein has its own characteristics with respect to the processes involved. Indeed, all individual types of protein synthesized and secreted by a given cell may differ in these respects, since other investigators have observed differ-
ent secretion kinetics for a variety of proteins in addition to those studied in the present work (3, 6, 7, 29, 30).

The cellular fractionation studies (Fig. 3) show that the general intracellular pathway for the synthesis and secretion of transferrin and α1-antitrypsin is the same as that of albumin (25, 31) and believed to be characteristic of secreted proteins, viz. synthesis on the rough endoplasmic reticulum and Golgi complex before secretion into the extracellular medium. The results confirm in a more quantitative fashion the reports that the delay in secretion of labeled transferrin in vivo occurs in the rough microsomal fraction (4) or in cell cultures in the total endoplasmic reticulum (5–7). We also found that this delay results in an actual accumulation of transferrin in the rough microsomes (Table I).

The slightly slower rate of secretion of α1-antitrypsin than of albumin may also be attributed to delayed passage from the rough endoplasmic reticulum, but the delay is far less than that found with transferrin. Another difference between albumin on the one hand and transferrin and α1-antitrypsin on the other is the relatively high level of radiolabeled and total albumin in the Golgi fraction compared with the values for the other two proteins. This suggests that albumin is limited in its rate of transport through the Golgi complex to a greater degree than is transferrin or α1-antitrypsin by the process of cleavage of proalbumin. However, any resultant decrease in secretion rate is small when compared with the much slower rate of transport of transferrin out of the rough endoplasmic reticulum than of albumin.

We sought to learn whether there is a delay in the early glycosylation steps of transferrin processing, both by measuring the kinetics of mannose incorporation in vivo and by tests of lectin binding by newly formed transferrin isolated from rough microsomes. Labeled mannose appeared equally promptly in both transferrin and α1-antitrypsin in the rough endoplasmic reticulum in vivo (Fig. 4). The level of incorporation of label into transferrin was only about one-third of that into α1-antitrypsin, but this is roughly in proportion to the relative carbohydrate contents of the two proteins, approximately 6% for transferrins (11) and 21% for α1-antitrypsin (17, 32). After a low level of incorporation during the first 5 min, there was a relatively steady rate of increase in the [3H]mannose content of both proteins during the remainder of the 30-min period of study. The difference from the pattern of [14C]leucine incorporation, which started within 1 min and was falling by the end of the experimental period, is probably a consequence of slower turnover of labeled mannose than leucine in the precursor pools as observed by Friesen and Jamieson (33).

In transferrin isolated from rough microsomes at various times after [3H]mannose and [14C]leucine injection (Table II), the proportion of labeled transferrin which could be bound by concanavalin A-Sepharose was constant and at a high level after the first 5 min (approximately 97% of the total). At no time was there any difference between the percentages of the [3H]mannose and [14C]leucine labels incorporated into transferrin which bound to the concanavalin A-Sepharose. If there was a delay in the initial incorporation of mannose into newly synthesized transferrin molecules, it would be expected that a lower percentage of the [14C]label would have been bound by the affinity column at the early time points. Overall, these experiments demonstrate equally rapid incorporation of mannose into transferrin and α1-antitrypsin, and they provide evidence that glycosylation does not delay export of transferrin from the rough endoplasmic reticulum. However, it is possible that the minor delay in α1-antitrypsin secretion relative to that of albumin and a small fraction of the delay with transferrin is due to the processes involved in glycosylation of these proteins.

The role of glycosylation in plasma protein synthesis has also been investigated by the use of specific inhibitors of this process (29, 30, 34–36). The secretion of α1-antitrypsin is more sensitive than that of transferrin to the action of inhibitors such as tunicamycin, implying that the oligosaccharide chains of α1-antitrypsin but not of transferrin are required for the intracellular transport of the proteins. Moreover, inhibition of glycosylation of transferrin does not accelerate its secretion as might be expected if the normal slow secretion was a consequence of the time required for the addition and processing of the carbohydrate chains on the protein. These considerations support the concept that the major delay in transport of transferrin out of the rough endoplasmic reticulum is unrelated to the glycosylation process.

The lower [3H]mannose content of both transferrin and α1-antitrypsin in smooth endoplasmic reticulum and Golgi fractions of the liver than in the rough endoplasmic reticulum (Fig. 5) is consistent with current views on the biosynthesis of glycoproteins. That is, high-mannose type oligosaccharide...
chains are added to the polypeptides in the rough endoplasmic reticulum at an early stage after their synthesis. These Endo H-sensitive chains are then processed to complex type, Endo H-resistant chains by removal of mannose and addition of other carbohydrates while the proteins traverse the smooth endoplasmic reticulum and Golgi complex (37). Nakada et al. (4) have also observed a reduction in [3H]mannose content of transferrin as it moves through these organelles. They showed that the oligosaccharide chains of transferrin remained sensitive to Endo H, even in the Golgi fractions of the cells, and that the glycans did not become fully resistant to the enzyme until just before secretion.

Transfer of a protein molecule from the rough endoplasmic reticulum may depend on the attainment of disulfide bridging and of a certain level of organization of its tertiary structure, processes which may take longer in the case of transferrin than of other secreted proteins. In the case of albumin, disulfide bonding is apparently completed within 1 min of release of the nascent peptide chain from the polysome (22). The disulfide bonds of transferrin, however, are not in orderly sequence as are those of albumin (12), but overlap heavily within the two domains of the transferrin chain, in one domain and 11 in the other (11). Hence, SS bridging of transferrin would be a complex process and might require considerable time.

Upon rapid alkylation with iodoaceticamide in vivo, we found that there are only 1 to 2 residues of the 38 Cys/2 per transferrin molecule in the rough endoplasmic reticulum which are in the thiol form. Nor could mixed disulfides such as cysteine-S-S-glutathione or cysteine-S-S-cysteamine be detected. Hence, intrachain disulfide bonding apparently occurs with about the same rapidity as it does for albumin and L chain (22, 38), within 1–2 min of chain completion.

Completion of disulfide bonding does not mean that native configuration has been obtained, since proteins during their folding are known to form mismatches which are then rearranged through catalysis by microsomal disulfide isomerase (39). Considering the complexity of transferrin SS bridging, mismatching would be more likely with this protein than with albumin or L chain, which have sequential SS bonds. We tested for native configuration of rough microsomal transferrin by observing its proteolytic peptide pattern and its binding properties for iron and for the transferrin receptor on red cell membranes.

The patterns of large peptides obtained by limited proteolysis of an unreduced protein give a measure of its disulfide bonding pattern. Transferrin from serum and transferrin from rough microsomes showed similar peptide patterns with three proteases (Fig. 6). Although this test is but a sampling technique, it readily showed differences in transferrin with 2.6 of 38 Cys/2 residues alkylated. The results therefore suggest that transferrin rapidly attains its native bonding configuration in the rough endoplasmic reticulum. This is supported by the observations that rough microsomal transferrin is able to bind iron, to interact with reticuloocyte receptors, and to donate iron to the cells in an almost identical manner with that of plasma transferrin. It was shown previously that removal of sialic acid from transferrin does not affect these properties (40). In addition, the ability to isolate full-size labeled transferrin molecules from the rough microsomes after only 5 min of labeling with leucine by precipitation with antibodies shows that the molecule has formed at least two functional antigenic determinants by that time. Spontaneous folding of incompletely transferrin molecules during the steps of isolation from liver seems improbable since transferrin, unlike albumin (41), does not refold spontaneously to yield native properties such as binding of iron, binding of antibodies, or binding to reticuloocytes following reduction of its disulfide bonds in vitro.

The question may be asked whether the transferrin in rough microsomes includes a significant amount of mature transferrin taken up from the plasma into endocytotic vesicles. Several lines of evidence indicate that the protein is indeed primarily newly formed transferrin. 1) The amount of transferrin measured in the rough microsomes, 73 µg/g liver (Table 1), is in agreement with the amount which would accumulate in this organelle in 30 min at the observed rate of transferrin biosynthesis of 140 µg/g liver·min−1 (1), i.e. 70 µg. 2) The endocytotic vesicles in which plasma transferrin is taken up into liver have been found to sediment at a density of 1.10 to 1.26 g/cm3, and hence would not be present in either of the endoplasmic reticulum fractions isolated as described under Cell Fractionation (see Miniprint). 3) Only 3% of the isolated rough microsomal transferrin was traceable to circulating125I-transferrin which had been injected intravenously before preparing liver cell fractions. 4) The glycan chains on the isolated rough microsomal transferrin were uncharged and were sensitive to removal by Endo H, properties of high-mannose N-linked chains, but not of mature oligosaccharide chains (4, 6).

The above studies suggest that neither glycosylation nor the disulfide bonding process is responsible for the delay in transferrin transport in the rough endoplasmic reticulum. Other causes for the characteristic delays for individual proteins may lie in their transport mechanisms. Each may be exported on its own carrier, several might compete for a common carrier as suggested by Fries et al. (7), or there may be differences in interactions with the membrane of the rough endoplasmic reticulum.

Transferrin receptors are involved in the receptor-mediated endocytosis of transferrin and cellular uptake of iron. This process has been studied in greatest detail in immature erythroid cells, in which both transferrin and its receptor recycle from the cell surface to intracellular sites without degradation with an average recycling time of 3–4 min (24, 42). Recycling kinetics of transferrin receptors in hepatocytes have recently been observed to be similar to those found with erythroid cells. Endocytosed transferrin is localized in low density vesicle fractions of hepatic cells (density 1.10–1.16 g/cm3) similar to the Golgi fractions isolated in the present work. Here the iron is released from the transferrin before the protein is recycled to the plasma. This and the present study suggest that both newly synthesized transferrin and endocytosed plasma transferrin pass through the Golgi complex before leaving the hepatocyte by exocytosis. It will be of interest to determine whether the two pathways share the same intracellular organelles and, perhaps, even the same transferrin receptors.

Ara and Papaconstantinou (43), in explaining their observations that transferrin transit time increases markedly in aged mice but is restored to normal by dexamethasone, considered that transferrin receptors might function in intracellular transport of newly formed transferrin. If this mechanism was applicable, the delay and accumulation of transferrin in the rough endoplasmic reticulum could result from a backlog of transferrin molecules awaiting loading onto a rate-limited transport apparatus. It does not, however, account for the rapidity of transferrin secretion in analbuminemia (13).

The rate of transferrin synthesis is about 1/6 that of albumin.

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3 T. Peters, Jr., unpublished experiments.
4 E. H. Morgan, G. D. Smith, and T. J. Peters, manuscript submitted for publication.
5 D. Trinder, E. Baker, and E. H. Morgan, submitted for publication.
et al. (6)). If transferrin and albumin compete for a common, capacity-limited carrier such as the receptor suggested by Lodish et al. (6), with albumin having a 6-fold greater affinity for the carrier, it might be expected that transferrin would accumulate in the site of its synthesis until its concentration there equaled that of albumin so that it could compete for 1/6 of the transport sites. Indeed, the concentration of transferrin in the rough endoplasmic reticulum does nearly equal that of albumin (Table 1). This mechanism would also be in accord with the observation that transferrin secretion is accelerated in analbuminemic rats, and the amount of intracellular transferrin in their livers is diminished (13).

One purpose in studying the effect of cycloheximide on the secretion of proteins prelabeled with $^{3}H$-leucine was to investigate this hypothesis. Previously, cycloheximide was shown not to interfere with the secretion of preformed serum albumin and the intracellular pool of this protein to become depleted during the 30 min following injection of the inhibitor (44). Since transferrin is secreted more slowly than albumin, the ratio of intracellular albumin:transferrin should fall during this period so that there would be relatively less albumin to compete with transferrin for a common transport mechanism in the rough endoplasmic reticulum. Therefore, if this hypothesis is correct, the transferrin secretion rate should accelerate after the administration of cycloheximide. However, the transferrin secretion rate did not change. Hence, the results suggest that transferrin and albumin do not compete in their secretion and that competition does not explain the reported acceleration of transferrin secretion in analbuminemic rats (13).

The relative binding of transferrin and albumin to rough microsomal membranes was tested, but differences were insignificant. The available methods for such testing are insensitive, however, so that some tighter binding of transferrin cannot be excluded.

In summary, the major contributions of this paper are 1) to quantitate the delay of transferrin release from the rough endoplasmic reticulum in vivo and show that it is paralleled by an increase in the amount of immunologically detectable transferrin in that organelle; 2) to show that glycosylation of transferrin begins promptly in the rough endoplasmic reticulum and that no aglycotransferrin is detectable; and 3) to show that disulfide bonding and tertiary folding are rapid processes, occurring within a few (1–2) minutes of peptide chain formation. Exclusion of the glycosylation and folding steps as causes of delay increases interest in some form of receptor mechanism for transferrin transport.

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**Biosynthesis of Rat Transferrin**

**EXPERIMENTAL PROCEDURE**

**Materials** — From Sigma were purchased, micrococcal, digest, o-methionine, phenylthio-carbamyl fluorophore, glutamic, and the anti-transferrin (C) rabbit polyclonal serum. For these measurements the sera of rabbits with complete Freund's adjuvant at 10-14 day intervals. The rabbits were bled by electrophoresis with immunofixation.

**Pronase,** activity was determined at appropriate times. Blood samples were collected in tubes containing 15 uCi of [125I]leucine, and antibody was added at the rate of 1:100,000. The antibodies were precipitated from the supernatant of 15 uCi, and the precipitate was washed with acetone.

**Assay of Radioactivity** — The assay was performed by adding radiolabelled transferrin and non-labelled transferrin (100 ng) to each well of a 96-well plate. The samples were incubated at 37°C for 16 h. The amount of radiolabelled transferrin bound to the antibody was measured by a scintillation counter.

**Preparation of Transferrin and Assay** — The transferrin solutions were dialysed against phosphate-buffered saline, with 0.15 M NaCl and 0.05 M sodium phosphate buffer, pH 7.4. The transferrin was assayed using a protein assay kit.

**Elution of Transferrin from Agarose** — Transferrin was eluted from the agarose column with 0.5 M NaCl. The eluate was collected and assayed for radioactivity.

**Immunoprecipitation** — Immunoprecipitation was performed using rabbit anti-transferrin serum and protein A-agarose. The precipitate was then washed and resuspended in 0.5 M NaCl.

**Electron Microscopy** — The transferrin fractions were fixed with 2.5% glutaraldehyde and postfixed with 1% OsO4. The samples were embedded in Epon-Araldite and sectioned for electron microscopy.

**RESULTS**

**Biosynthesis of Rat Transferrin** — The biosynthesis of rat transferrin was studied using [125I]leucine incorporation and immunoprecipitation.

**Immunofixation** — Immunofixation was performed using rabbit anti-transferrin serum and protein A-agarose. The precipitate was then washed and resuspended in 0.5 M NaCl.

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Analytical Methods — Protein in cell fractions and in immune precipitates was measured by an automated Lowy procedure (25) using bovine serum albumin as standard. Antiproteic activity was measured as described by Dietz et al. (26). Techniques for hydrolysis of proteins, amino acid analysis and electrophoresis on cellulose acetate and polyacrylamide slab gels were as described previously (25). Electrophoresis slab gel analysis, western blots to nitrocellulose sheets and staining of proteins with amido black were performed according to Towbin et al. (27). Detection of glycans chains with ConA and peroxidase was performed on blots by the method of Kikimoto-Ochiai et al. (28), with a-dichloro-l-naphthol as color reagent. Total protease-bound radioactivity was determined by precipitating with cold 0.5 Tritonx-100 acid and washing the precipitated 3 times with the same reagent. 14C and 3H radioactivity was counted in a Beckman liquid scintillation counter using Liquiscint (National Diagnostics, Somerville, NJ) as the scintillation solution. Observations were made by the scintillation "Bumber" method. Efficiency was about 91% for 14C and 86% for 3H. 1% was counted on an Albutt well counter with an efficiency of about 92%.

RESULTS

Time Course of Leucine Incorporation and the Efect of Cycloheximide — Incorporation of [3H]leucine into plasma proteins and the effect of cycloheximide — separated plasma samples were sampled between 15 and 180 min after the intravenous injection of [3H]leucine and the incorporation of radioactivity into transferrin, albumin, and α-antitrypsin measured. Half of the rats were injected with cycloheximide (300 mg/100 g body weight intravenously and 200 mg/100 g body weight proportionally) 10 min after administration of the [3H]leucine. In a control experiment it was shown that this dose of cycloheximide, given immediately before injecting [3H]leucine, reduced the incorporation of the label into transferrin and albumin to less than 1% of the control in rats without cycloheximide. However, it was found that the inhibitor given at this time had no effect on the incorporation of [3H]leucine into any of the secreted proteins (Fig. 1A). Hence, the results for the control rats and the rats given cycloheximide were combined and are illustrated in Fig. 1B.

FIG. 1. Incorporation of [3H]leucine into plasma proteins: A. Fate of leucine (3.5 mg/100 g) (3) or without (C) The injection of cycloheximide 10 min after the [3H]leucine.
B. Fate of leucine (3.5 mg/100 g) albumin (4) and α-antitrypsin (5). The values in A are the means from 3 animals. As with transferrin, cycloheximide was found to have no significant effect on the incorporation of [3H]leucine into albumin or α-antitrypsin. Therefore, in A, the data from all 4 animals has been combined. The vertical bars represent the SEM.