# The Effects of Structural Modifications on the Biologic Activity of Human Transferrin*

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ABSTRACT: The effects of various structural modifications of human transferrin on its biologic activity have been studied by measuring three functions of the protein: (1) the iron-binding capacity of the molecule, (2) the binding of the protein to reticulocytes, and (3) the ability of the molecule to transfer iron to reticulocytes. By considering the reticulocyte–iron–transferrin interaction to be analogous to an enzyme–substrate reaction obeying Michaelis–Menten kinetics, the experimental data have been analyzed to yield apparent affinity constants of reticulocytes for these modified transferrins. Acetylation and carbamylation of up to 25% of the available amino groups results in a minimal decrease in iron-binding capacity. There is a marked stimulation of binding to noniron-transfer sites on the reticulocyte cell surface while the affinity for the physiologic iron-transfer sites is greatly impaired. The maximal rate of iron release to the cells is only slightly altered. Amidinated transferrin (84% modified), with an unaltered net charge, functions normally except for mild impairment of binding to iron-transfer sites. When transferrin is trinitrophenylated, binding to noniron-transfer sites occurs after 15% reaction while the iron-binding capacity does not decrease until 75% reaction. Extensively trinitrophenylated transferrin has an apparently normal binding affinity for iron-transfer sites on the cell surface and maximal iron transfer is greater than normal. However, the kinetic data, including competition experiments, suggest that there is a complex interaction of this modified transferrin with the reticulocytes. These studies demonstrate that the iron-binding capacity of transferrin is relatively insensitive to blockage of free amino groups and to the net charge of the protein. Binding of transferrin to reticulocyte iron-transfer sites is affected by both these parameters with net charge alterations being more important. Iron release to reticulocytes is normal or accelerated even with extensive protein modifications.

The enzymatic removal of most of the carbohydrate molecules of the two heterosaccharide chains of transferrin causes no significant alteration in the biologic functions tested.

Transferrin, an iron-binding plasma glycoprotein with a molecular weight of approximately 90,000, functions primarily in the transport of iron from sites of iron absorption and storage to immature red blood cells (Jandl *et al.*, 1959; Morgan and Laurell, 1963; Schade, 1964). The latter process involves first the binding of the transferrin molecule to the immature erythrocyte surface followed by the transfer of the iron to the cell and then the release of transferrin from the cell (Morgan and Laurell, 1963; Jandl and Katz, 1963; Morgan, 1964). Little is known about the structural determinants of the transferrin molecule that enable it to carry out these various functions. Several genetic variants of transferrin, presumably due to amino acid substitutions (Wang and Sutton, 1965), apparently function normally both in their ability to bind iron and to transfer it to immature red blood cells (Turnbull and Giblett, 1961).

Transferrin free of sialic acid appears to behave in a normal fashion (Morgan *et al.*, 1967). Buttkus *et al.* (1965) found that considerable alteration of the free amino groups of transferrin could be carried out without affecting the capacity of the molecule to bind iron. The ability of these altered transferrins to transfer iron to immature red blood cells was not tested.

The present study was performed to determine the effects of structural modifications of human transferrin on its ability to bind iron and to transfer it to immature red blood cells. Chemical modifications of the free amino groups of the molecule were carried out with a number of reagents. Also, most of the carbohydrate of the two heterosaccharide chains (Jamieson, 1965) of transferrin was enzymatically removed. Following these treatments the biologic activity of the altered transferrins was tested.

## Experimental Procedure

### *Materials*

Purified human iron-free transferrin and *Vibrio cholerae* neuraminidase were obtained from Behringwerke, Marburgh-Lahn, Germany. Ethyl acetimidate–HCl was a gift from Dr. Charles Parker. Trinitrobenzenesulfonic acid was purchased from Pierce Chemical Co., Rockford, Ill. Jack bean meal was obtained from Sigma Chemical Co., St. Louis, Mo. All other chemicals used were analytical reagent grade.

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*Methods*

*Rabbit Reticulocytes.* Reticulocytosis was produced in adult rabbits by repeated bleedings at 2–3-day intervals. The blood was collected with heparin as anticoagulant, centrifuged to remove the plasma and buffy coat, and then washed three times with cold 0.9% sodium chloride buffered with 0.01 M sodium bicarbonate. The reticulocyte count was 15–30% in most of the experiments. For convenience, these reticulocyte-rich red blood cell suspensions will be referred to as "reticulocytes."

*Chemical Modifications.* ACETYLATION. Transferrin was acetylated as described by Buttkus *et al.* (1965). Purified, human, iron-free transferrin (200 mg) was dissolved in 3.9 ml of 50% saturated sodium acetate (pH 8.2). To this solution was added 0.14 ml of ferric chloride (10 mg/ml). The reaction mixture was mixed well and allowed to incubate at 37° for 10 min prior to chilling to 4°. The solution was then divided into four 1.0-ml aliquots. One aliquot ("control") was diluted with 9.0 ml of cold $H_2O$ and then dialyzed against 2 l. of 0.9% NaCl–0.01 M $NaHCO_3$. To the other aliquots, acetic anhydride was added as follows: 0.5 $\mu$l for 3 min, 1 $\mu$l for 3 min, and 3.0 $\mu$l for 4 min. These samples were then diluted with 9.0 ml of $H_2O$ and dialyzed against the same buffer in the cold. After 5 hr, the dialyzing medium was changed to 0.025 M citrate buffer (pH 4.6) in order to remove the iron from the transferrins. After 20 hr, the samples were redialyzed for 20 hr against 0.9% NaCl–0.01 M $NaHCO_3$. The final transferrin solutions were perfectly clear with no visible precipitate.

CARBAMYLATION. The conditions for carbamylation were those of Buttkus *et al.* (1965). Transferrin (180 mg) was dissolved in 20 ml of 0.05 M Tris-HCl buffer (pH 8.0); 0.13 ml of $FeCl_3$ (10 mg/ml) was added and the solution was incubated for 10 min at 40°. A 5.0-ml aliquot was removed (control) and 1.0 ml of freshly prepared 1.0 M KCNO was added to the reaction mixture. Other 5.0-ml aliquots were removed at 10, 30, and 360 min. The aliquots were chilled to 4° and then dialyzed against 3 l. of 0.9% NaCl–0.01 M $NaHCO_3$. The iron was removed from the transferrin as described in the previous paragraph.

AMIDINATION. The amidination of transferrin was carried out by the method of Wofsy and Singer (1963). Transferrin (20 mg) was dissolved in 2.0 ml of 0.1 M borate buffer (pH 8.5). To the transferrin was added 0.015 ml of $FeCl_3$ (10 mg/ml), and the solution was incubated at 37° for 10 min and then chilled to 4° in an ice bucket. Ethyl acetimidate–HCl (50 mg) plus 0.025 ml of 5 N NaOH were then added to the reaction and the pH was adjusted to 8.4 with more NaOH. After 1 hr, another 50 mg of ethyl acetimidate was added, the pH was readjusted to 8.3, and the reaction was allowed to continue for another hour with constant stirring. A control sample was prepared in borate buffer to which no ethyl acetimidate was added. After 2 hr, each reaction mixture was dialyzed as previously described.

TRINITROPHENYLATION. Transferrin (63.5 mg) was dissolved in 6.0 ml of 4% $NaHCO_3$ (pH 8.5) and then divided into two equal portions. To one portion was added 41 $\mu$g of iron (containing 15 $\mu$Ci of $^{59}FeCl_3$) followed by a 20-min incubation at room temperature. A control aliquot (0.55 ml) was removed from each reaction mixture and then 16 mg of solid trinitrobenzenesulfonic acid was added to each of the reaction mixtures. At 15, 30, 60, and 180 min, aliquots of 0.55 ml were removed. A portion (0.05 ml) was added to 3.0 ml of 4% $NaHCO_3$ (pH 8.5) followed by the addition of 1.0 ml of 10% sodium dodecyl sulfate and 0.5 ml of 1.0 N HCl. The absorbancy of the solution at 344 m$\mu$ was determined using as a blank a similar solution which contained 0.05 ml of $H_2O$ rather than protein. By this method the rate of the reaction could be followed and the number of free amino groups that had reacted could be quantitated (Habeeb, 1966).

The other 0.5 ml of the aliquot was passed through a small Amberlite IRA-400 column to remove the unreacted trinitrobenzenesulfonic acid. The column was washed with 0.5 ml of 0.9% NaCl–0.01 M $NaHCO_3$ and the trinitrophenylated transferrin samples were kept at 4° until used.

*Iodination of Transferrin.* Iron-free transferrin was iodinated by the method of Awai and Brown (1963).

*Determination of Free Amino Groups.* Except in the case of the trinitrophenylation experiments, free amino groups were determinated by the ninhydrin method (Fraenkel-Conrat, 1957). The per cent of amino groups modified was calculated from the difference between the free amino groups present in the original and the modified proteins.

*Determination of Iron-Binding Capacity.* When iron is added to transferrin, a red complex is formed that has a maximum absorption at 465 m$\mu$. This property ("chromogenic capacity") can be used to determine the iron-binding capacity of a transferrin solution. $FeSO_4$ (0.01 M) was added to approximately 2.0 mg of transferrin in 1.0 ml of 0.9 NaCl–0.01 M $NaHCO_3$ until the iron-binding capacity of the molecule was saturated. The complex was incubated at 37° for 20 min and the optical density was determined at 465 m$\mu$. A 1:10 dilution of the solution was then made and the optical density was determined at 280 m$\mu$. The intense absorption of the trinitrophenylated transferrin derivatives made this method unsatisfactory so an alternative method was used to determine the iron binding of these derivatives. Control and trinitrophenylated transferrins were allowed in incubate with $^{59}Fe$ solutions and the samples were passed over Sephadex G-25 columns (1 × 10 cm). The excluded material (containing the transferrin-bound $^{59}Fe$) was collected and an aliquot was counted in a Packard Autogamma well counter. The number of counts bound by each sample of transferrin was compared to that bound by an unaltered control.

*Starch Gel Electrophoresis.* Vertical starch gel electrophoresis was carried out by the method of Smithies (1955).

*Enzymatic Removal of Carbohydrate from Transferrin.* The glycosidases $\beta$-galactosidase, $\alpha$-mannosidase, and $\beta$-*N*-acetylglucosaminidase were prepared from jack bean meal essentially as described by Li (1966). In some experiments the dialyzed 33–50% ammonium sulfate fraction was used as a source of enzyme while in others

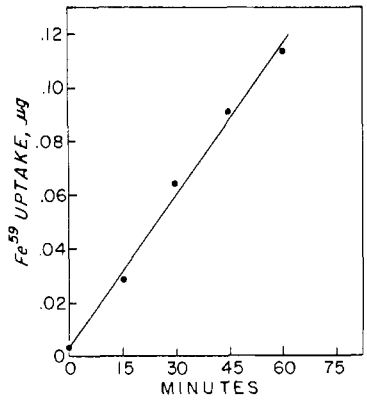


FIGURE 1: Effect of the time of incubation on $^{59}Fe$ uptake from human transferrin by rabbit reticulocytes. Standard assay conditions.

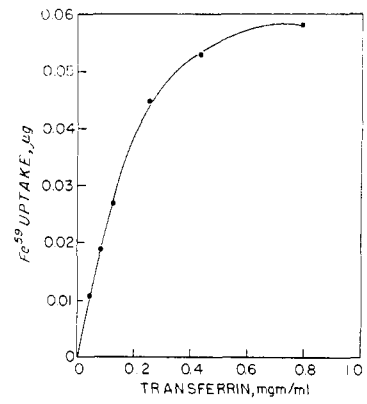


FIGURE 2: Effect of human [$^{59}Fe$]transferrin concentration of $^{59}Fe$ uptake by rabbit reticulocytes. The incubation time was 30 min.

the more highly purified fractions obtained from gel filtration on columns of Bio-Rad P-200 were used. Both the neuraminidase preparation and the 33–50% ammonium sulfate fraction of the jack bean enzyme were free of proteolytic activity as measured by the method of Anson (1938). The removal of the carbohydrate from the transferrin was carried out in two steps. A typical experiment was as follows. Purified *Vibrio cholerae* neuraminidase (300 units) was added to a reaction mixture (4.6 ml) containing 50 mg of transferrin, 5 μmoles of $CaCl_2$, and 200 μmoles of phosphate–citrate buffer (pH 6.2). The reaction mixture was incubated at 37° under a toluene atmosphere and at various times 50-μl aliquots were removed and analyzed for unbound sialic acid by the method of Warren (1959). At 6 and 12 hr, additional neuraminidase was added to the reaction. After 48 hr virtually all of the bound sialic acid had been released. The total sialic acid content of transferrin was determined following acid hydrolysis at pH 2 for 1 hr at 80°. The neuraminidase-treated reaction mixture was dialyzed against a large volume of cold water. A portion of the sialic acid free transferrin (10 mg) was then added to a reaction mixture containing 40 μmoles of citrate buffer (pH 4.6) and 3 mg of the jack bean 33–50% ammonium sulfate fraction in a final volume of 2.0 ml. The reaction mixture was incubated at 37° and after 3–6 hr additional enzyme was added. At various times, 0.1-ml aliquots were removed from the reaction mixture and the bound sugars were precipitated with equivalent amounts of $Ba(OH)_2$ and $ZnSO_4$. Following centrifugation, the clear supernatant solutions were assayed for carbohydrate content by the reducing sugar method of Nelson (1944). The enzymatic treatment was usually carried out for 7–9 hr at which time the remaining reaction mixture was dialyzed against a large volume of 0.01 M $NaHCO_3$ in the cold. In one experiment, the enzymatically released sugars were identified by concentrating the dialysate, deionizing the solution by passage through an Amberlite MB-3 mixed-bed resin, and then chromatographing the neutral sugars on Whatman No. 1 paper using a solvent system of butanol–ethanol–$H_2O$ (10:1:2).

*Assay of Transferrin Binding and Iron Transfer to Reticulocytes.* The usual assay method was that described by Jandl *et al.* (1959). Transferrin (2.62 mg/ml dissolved in 0.9% NaCl–0.01 $NaHCO_3$) was mixed with an equal volume of 20 μM $^{59}FeSO_4$ to give one-third saturation of the iron-binding capacity of the protein. The solution was then incubated at 37° for 20 min prior to use. The usual reaction mixture contained 0.5 ml of reticulocytes, 0.5 ml of 0.9% NaCl–0.01 M $NaHCO_3$, and 0.25 ml of the [$^{59}Fe$]transferrin solution. Following incubation for 30 min at either 0 or 37° the reticulocytes were washed three times with seven volumes of cold saline and then suspended in a final volume of 5.0 ml with the same buffer. The suspension (2 ml) was counted in a Packard Autogamma well counter in order to determine the total uptake of radioactivity. The remaining three milliliters of cell suspension were sedimented and the packed cells were lysed with ten volumes of cold water. The lysate was centrifuged at 15,000*g* for 15 min and the amount of radioactivity in the supernatant fluid was determined. This value was used as a measure of $^{59}Fe$ transfer into the cell. Usually most of these counts were present in heme as determined by the method of Fischer (1941). When the reactions were carried out at 0° no counts appeared in the 15,000*g* supernatant solution, so these reactions were a measure of [$^{59}Fe$]transferrin binding to the cell surface.

Another method employed to determine transferrin binding to reticulocyte membranes involved addition of [$^{59}Fe$]transferrin to the cells in an ice bucket, rapid mixing, and immediate centrifugation of the suspension. Under these circumstances, virtually all of the $^{59}Fe$ taken up by the cells was membrane bound, so that the radioactivity of each sample could be compared directly to the control.

The dependence of the assay on time is shown in Figure 1. The effect of varying the amounts of transferrin and reticulocytes in the reaction mixture is shown in Figures 2 and 3.

## Results

*Carbamylation Experiments.* Table I shows the effect of carbamylation of up to 23% of the available free amino groups of transferrin. Under the conditions used, there was only a minimal effect on the "chromogenic

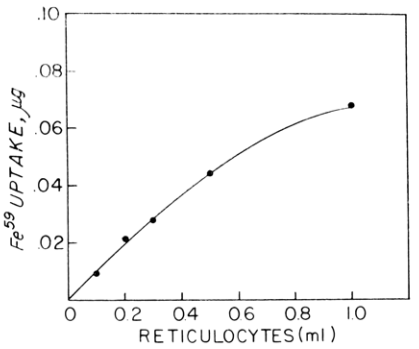


FIGURE 3: The relation between reticulocyte concentration and uptake of $^{59}Fe$ from [$^{59}Fe$]transferrin. The standard assay system was used except that the volume of the reaction mixture was 1.5 ml.

capacity" of the molecule as evidenced by the slight increase in the 280–465 ratio of the sample. This measurement suggests that iron binding by the altered transferrins is almost normal, a finding in agreement with the results of Buttkus *et al.* (1965). Starch gel electrophoresis of these samples (Figure 4) shows that the minimally modified transferrins run slightly faster anodally than the control samples; the more extensively carbamylated sample runs well ahead of the others and shows some heterogeneity as evidenced by spreading on the gel. When these transferrins were tested for their ability to bind to reticulocytes and to transfer iron, the results shown in Figure 5 were obtained. As the extent of carbamylation is increased, there is a striking increase in the binding of transferrin to the reticulocytes as compared to the untreated transferrin. On the other hand, in spite of this stimulation of binding, the incorporation of radioactive iron into heme is markedly depressed.

TABLE I: Acetylation and Carbamylation of Transferrin.

| Conditions[a] | | | Results | |
|---|---|---|---|---|
| Expt | Moles of Reagent/ Mole of Amino Groups[b] | Reaction Time (min) | % of Amino Groups Modified | *E* 280:465 mμ |
| | | Carbamylation | | |
| 1 | 0 | 0 | 0 | 23.8 |
| 2 | 12.0 | 10 | 4 | 24.2 |
| 3 | 12.0 | 30 | 7 | 24.0 |
| 4 | 12.0 | 360 | 23 | 25.1 |
| | | Acetylation | | |
| 1 | 0 | 0 | 0 | 23.2 |
| 2 | 0.34 | 3 | 10 | 24.9 |
| 3 | 0.68 | 3 | 18 | 25.5 |
| 4 | 1.03 | 4 | 25 | 26.2 |
| | | Amidination | | |
| 1 | | | 0 | 24.0 |
| 2 | | | 84 | 22.0 |

[a] See Methods for experimental details. [b] Based on presence of 54 moles of amino groups/mole of human transferrin (Buttkus *et al.*, 1965).

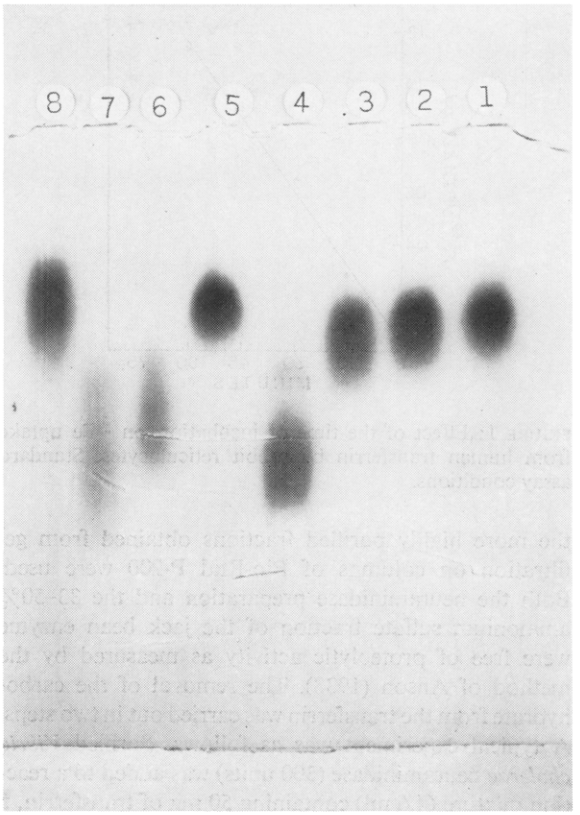


FIGURE 4: Starch gel electrophoretic patterns of carbamylated, acetylated, and amidinated transferrins. Samples 1, 2, 3, and 4 corresponding to carbamylation samples 1, 2, 3, and 4 of Table I are iron-containing transferrins which had been carbamylated for 0, 10, 30, and 360 min, respectively; sample 5 is the control iron transferrin; samples 6 and 7 are iron transferrins which had been aceylated for 3 and 4 min, respectively. These samples correspond to acetylated samples 3 and 4 of Table I; sample 8 is amidinated transferrin corresponding to the sample described in Table I. General conditions of these experiments are described in the text.

*Acetylation Experiments.* Treatment of transferrin with acetic anhydride to form acetylated derivatives of the free amino groups gave results very similar to those obtained after carbamylation. Table I shows that the chromogenic capacity of the transferrin was slightly more altered after acetylation than after carbamylation. Likewise, on starch gel electrophoresis (Figure 4) there is more spreading of the bands suggesting multiple components with somewhat different degrees of acetylation. When the biologic activity of these derivatives was tested (Figure 6), the results were similar to those found after carbamylation; that is, a marked stimulation of the binding of the altered transferrins to the reticulocytes but inability to transfer the radioactive iron into the cell.

*Trinitrophenylation Experiments.* When trinitrophenylation was carried out to a limited extent (less than 30% of the free amino groups blocked), the effects on the biologic activity of the molecule were limited to

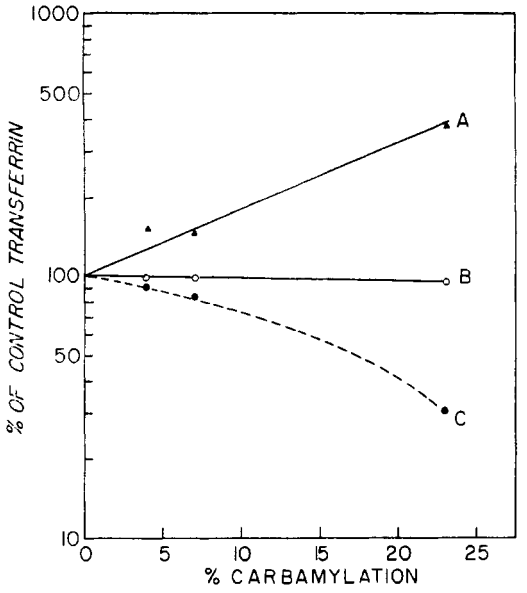


FIGURE 5: Effect of increasing carbamylation on the biologic function of transferrin. The carbamylation procedure and the assay method are described in the text. (A) Binding of [$^{59}$Fe]transferrin to reticulocytes; (B) binding of $Fe^{3+}$ to transferrin as determined by the optical density method (see text); (C) intracellular $^{59}$Fe incorporation. All results are expressed as per cent of the untreated control values.

the stimulation of transferrin binding to reticulocytes. Therefore, a more extensive trinitrophenylation was performed in which 75–100% of the free amino groups was blocked. The consequences of this modification are shown in Figure 7. The iron-binding capacity of the trinitrophenylated transferrins, as measured by the radioactive technique described in the Methods section, is only slightly decreased until approximately 85-

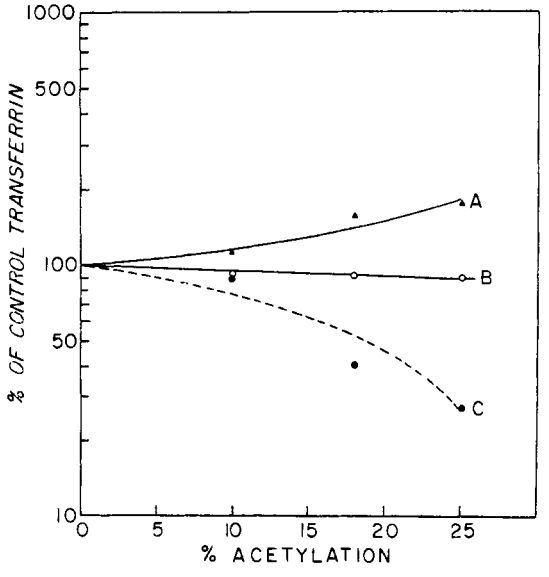


FIGURE 6: Effect of increasing acetylation on the biologic function of transferrin. The acetylation procedure and the assay method are described in the text. (A) Binding of [$^{59}$Fe]-transferrin to reticulocytes; (B) binding of $Fe^{3+}$ to transferrin as determined by the optical density method (see text); (C) intracellular $^{59}$Fe incorporation. All results are expressed as per cent of the untreated control values.

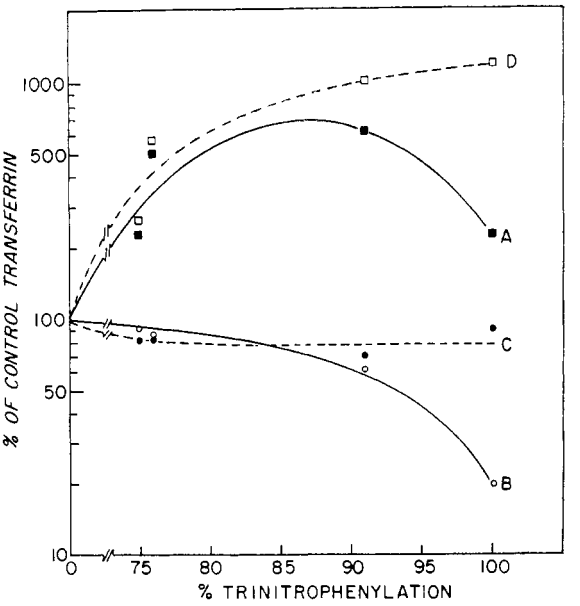


FIGURE 7: Effect of increasing trinitrophenylation on the biologic function of transferrin. The trinitrophenylation procedure and the assay method are described in the text. (A) Binding of [$^{59}$Fe]transferrin to reticulocytes expressed as total radioactivity bound; (B) binding of $Fe^{3+}$ to transferrin as determined by the gel filtration method (see text); (C) intracellular $^{59}$Fe incorporation; (D) binding of [$^{59}$Fe]-transferrin to reticulocytes expressed as the ratio of radioactivity bound to total radioactivity added per reaction mixture. All results are expressed as per cent of the untreated control values. Equal concentrations of transferrin were added to each reaction mixture. Therefore, the reaction mixtures containing the most extensively trinitrophenylated transferrin had less total radioactivity.

90% of the free amino groups is blocked at which point iron binding declines rapidly. At 75% trinitrophenylation, however, there is a marked stimulation of transferrin binding to reticulocytes, and as the transferrin is more modified there is a further increase in binding which levels off at 10- to 11-fold stimulation compared to control transferrin. In Figure 7, the binding of transferrin to the reticulocytes is plotted in two ways: as radioactivity bound expressed as per cent of the untreated control transferrin; and as the ratio of radioactivity bound to total radioactivity added to the reaction mixture (again compared to the control of 100%). Both plots are needed since in this experiment each reaction mixture contained the same amount of transferrin and in the case of the most modified transferrin, for example, only 20% as much radioactivity as the control transferrin. Thus, in the control reaction 335,000 cpm was added of which 555 cpm (0.16%) was bound to the reticulocyte membranes and 15,750 cpm (4.7%) was incorporated into heme. In the case of the 100% trinitrophenylated transferrin, 67,000 cpm was added to the reaction of which 1290 cpm (1.9%) was membrane bound and 14,100 cpm (21%) was incorporated into heme. In sharp contrast to the effect produced by acetylation and carbamylation, the incorporation of radioactive iron into heme is only slightly depressed even when there is complete blockage of the free amino groups of the transferrin molecule.

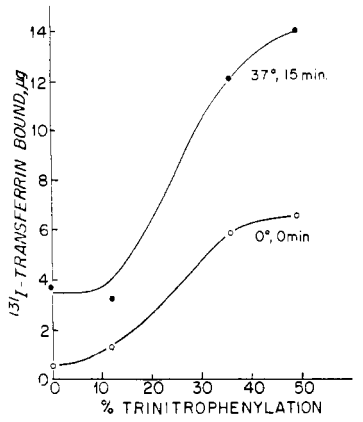


FIGURE 8: Effect of trinitrophenylation of [$^{131}$I]transferrin binding to reticulocytes. [$^{131}$I]Transferrin was trinitrophenylated as described in the text. The standard reaction mixture was used. One group of samples was incubated at 37° for 15 min while the other was centrifuged immediately after addition of the [$^{131}$I]transferrins to the precooled reaction mixtures. The reticulocyte suspensions were washed twice and the bound radioactivity was determined.

In another experiment, equal amounts of radioactivity (16,750 cpm) were added to each reaction mixture so that the control sample contained only 20% as much transferrin as the most altered sample. The results were essentially the same as before. In the reaction containing the control transferrin, 112 cpm (0.6%) was bound to the membranes and 2560 cpm (15.3%) was incorporated into heme. In the reaction containing the most altered transferrin, 990 cpm (5.9%) was membrane bound and 2310 cpm (13.8%) was incorporated into heme. In spite of the marked modification of the samples, on starch gel electrophoresis they ran as fairly discrete bands with very rapid mobilities.

*Direct Determination of Transferrin Binding to Reticulocytes.* In the previous experiments, the binding of transferrin to the reticulocyte cell surface was measured indirectly. To confirm the validity of this method, radioiodinated transferrin was prepared and aliquots were trinitrophenylated to various extents as described in the methods. The ability of $^{131}$I-labeled trinitrophenylated transferrins to bind to reticulocytes was then determined. As shown in Figure 8, trinitrophenylation resulted in a marked enhancement of transferrin binding both at 0 and 37°.

*Specificity of Transferrin Binding to Reticulocytes.* One explanation for the fact that carbamylation and acetylation of transferrin result in a decrease in iron transfer in spite of enhanced binding of the molecule to the cell surface is that the observed increase in transferrin binding is nonspecific and that, in fact, the binding of these altered transferrins to the "physiologic" iron-transfer sites on reticulocytes is actually impaired. Since direct measurements of transferrin binding to reticulocytes do not distinguish between nonspecific and specific (physiologic) sites, the ability of these modified transferrins to donate iron for heme synthesis was examined in more detail. In this way a measure of transferrin binding to physiologic iron-transfer sites was obtained. Figures 9 and 10 illustrate the effect of varying the concentration of the variously modified transferrins on intracellular iron uptake by reticulocytes. When these data were plotted by the method of Lineweaver and Burk (1934) the apparen affinity constant for unaltered transferrin varied between 0.08 and 0.024 mg per ml, depending on the particular batch of reticulocytes used for the experiment.[1] Because of this variability, assays of control transferrin were included in every experiment. Both the acetylated and carbamylated derivatives showed a marked increase in their apparent affinity constants compared to the unaltered transferrin. Further, the curves indicate that these modified transferrins have two main components which vary in their affinity for the iron-transfer sites. The calculated maximal velocity obtained with these transferrins is similar to the control demonstrating that they are able to release iron at a nearly normal rate once they are bound to the proper site on the reticulocyte cell surface. The trinitrophenylated transferrin, on the other hand, had an apparent affinity constant less than that of control transferrin and the maximal velocity is actually 35% greater than that obtained with control transferrin. Further, the experimental data obtained with this modified transferrin do not fall on a straight line.

*Inhibition of Normal Transferrin Function by Altered Transferrins.* To confirm that the modified transferrins were binding to the same receptor sites on the cell surface as untreated transferrin, competition experiments were carried out. Figure 11 shows the effect of various transferrins on the ability of normal transferrin to transfer iron to reticulocytes. The acetylated derivative was a weak inhibitor consistent with the fact that this modified transferrin has a relatively poor affinity for the physiologic iron-transfer sites. However, the trinitrophenylated transferrin did not inhibit as well as expected on the basis of its apparent affinity constant. Thus, at the 25-min point it had inhibited $^{59}$Fe uptake 55% while an equal amount of untreated transferrin inhibited 86%. Other experiments confirmed that the interaction of normal and trinitrophenylated transferrin is very complex and does not follow simple Michaelis–Menten Kinetics.

[1] The interaction of iron transferrin with reticulocytes has many similarities with an enzymatic reaction (Katz and Jandl, 1964). The reaction may be considered as follows.

$$\text{iron transferrin} + \text{reticulocyte} \underset{k_2}{\overset{k_1}{\rightleftarrows}} \text{transferrin–iron–reticulocyte} \xrightarrow{k_3} \text{transferrin} + \text{reticulocyte} + \text{intracellular iron (heme)}$$

In this sequence reticulocyte refers to the cell surface which is analogous to an enzyme, carrying out the catalytic function of transferring iron from transferrin into the cell where it accumulates, primarily in heme. By plotting intracellular $^{59}$Fe uptake as a function of [$^{59}$Fe]transferrin concentration, an apparent affinity constant for transferrin can be calculated. Since straight lines are obtained, the reaction appears to follow Michaelis–Menten kinetics. Modifications of transferrin can affect $k_1$, $k_2$, and $k_3$ and will be reflected in the determination of the apparent affinity constant and maximal velocity of the reaction. This analogy is obviously an oversimplication since each step of the reaction probably has many phases. However, analysis of the data in this manner serves to clarify a complex situation, and justifies the use of this model.

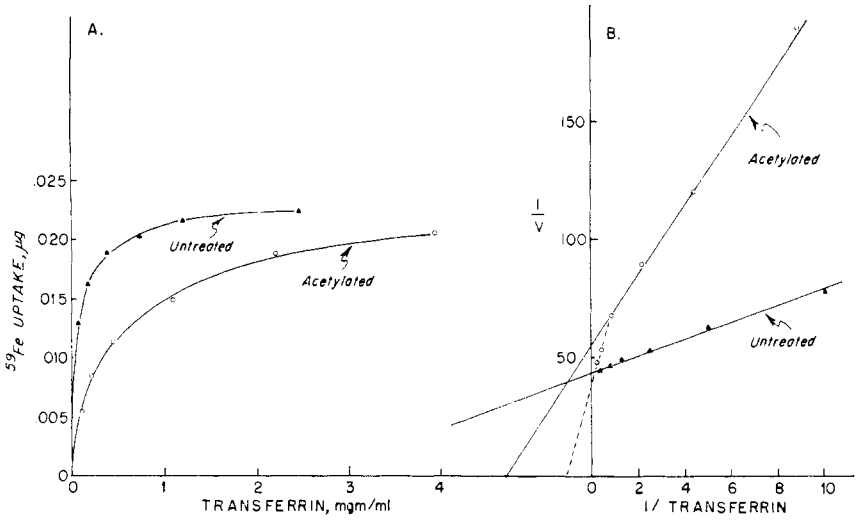


FIGURE 9: $^{59}$Fe uptake as a function of normal and acetylated transferrin concentration. Standard assay conditions were used with intracellular $^{59}$Fe uptake being determined. (A) Transferrin concentration (milligrams per milliliter) was varied as noted. The acetylated transferrin was 25% modified. (B) Double-reciprocal plot of the data in A with velocity ($V$) expressed as micrograms of $^{59}$Fe uptake in 30 min.

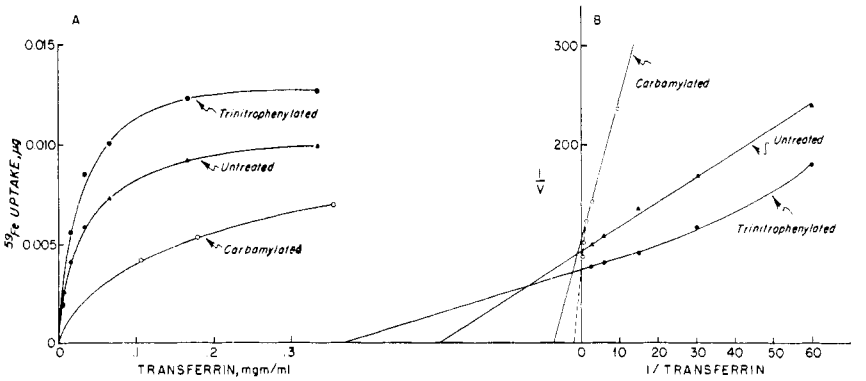


FIGURE 10: $^{59}$Fe uptake as a function of normal, carbamylated, and trinitrophenylated transferrin concentration. The experimental conditions are the same as those described in Figure 9. The carbamylated and trinitrophenylated transferrins were 23 and 66% modified, respectively.

*Transferrin Binding to Reticulocytes Compared to Adult Red Blood Cells.* Normal transferrin binds only to reticulocytes (Morgan and Laurell, 1963: Jandl and Katz, 1963). Since the above results suggest that much, if not all, of the enhanced binding of the altered transferrins is nonspecific, it was of interest to determine whether this enhanced binding took place with adult red blood cells as well as with reticulocytes. Control and trinitrophenylated transferrins were incubated at 0° with high- and low-percentage reticulocyte preparations and the binding to cell membranes was determined. Figure 12 demonstrates that both the control and altered transferrin bind much better to the surfaces of the cells with a high percentage of reticulocytes than to the cells with a low percentage of reticulocytes.

In other experiments using radioiodinated trinitrophenylated transferrin, it was shown that the affinity constants of this transferrin for binding to both iron-transfer and noniron-transfer sites were about equal.

*Amidination Experiments.* Each of the chemical modifications thus far discussed involves not only the blockage of the free amino groups of the molecule but also an increase in the net negative charge of the protein at neutral pH. (This change is reflected in the increased anodal mobility of the modified transferrins upon starch gel electrophoresis.) As a result, intramolecular electrostatic changes would be expected to occur with a concomitant change in the conformation of the molecule. The marked alterations in the biologic activity of these modified transferrins could be due either to blockage of key amino groups or to over-all conformational changes of the molecule. To examine this point directly, transferrin was treated with ethyl acetimidate hydrochloride, a reagent which reacts with free amino groups to form acetamidinium groups as shown in reaction 1.

$$-{}^{+}NH_3 \longrightarrow -NHCCH_3 \quad (1)$$
$$\qquad\qquad\qquad\quad \| \qquad\quad (1)$$
$$\qquad\qquad\qquad\quad {}^{+}NH_2$$

Consequently, the free amino groups of the molecule are blocked with no change in net charge of the molecule. The data in Table I show that 84% amidinated transferrin has a normal chromogenic capacity. When

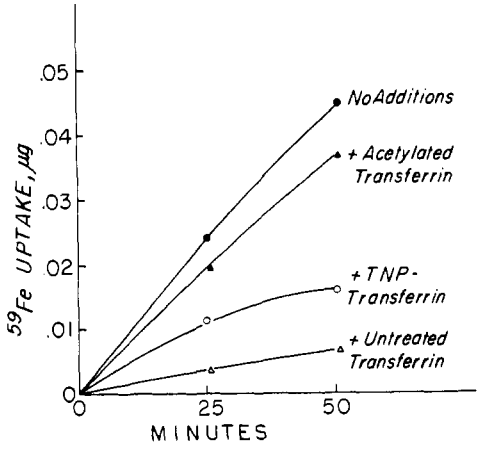


FIGURE 11: Inhibition of $^{59}Fe$ uptake from control transferrin by various transferrins. Each reaction mixture contained 0.12 mg of normal [$^{59}Fe$]transferrin and 1.2 mg of [$^{56}Fe$]-transferrin as noted. The acetylated transferrin was 25% modified and the trinitrophenylated transferrin was 66% modified.

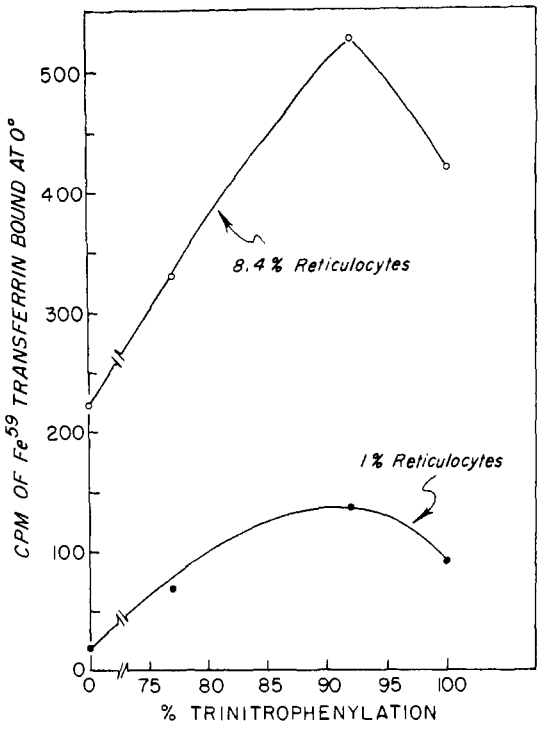


FIGURE 12: Effect of reticulocytes on the cell surface binding of normal and trinitrophenylated transferrin. The assay procedure was carried out in the usual fashion using [$^{59}Fe$]-transferrin except that the reaction mixture was kept at 0° during the time of incubation. Each transferrin was assayed with a cell suspension containing both a high and low percentage of reticulocytes.

its biologic activity was tested, it was found to bind normally to reticulocytes and to have an affinity constant for binding to iron-transfer sites that was threefold greater than normal transferrin. The starch gel electrophoretic mobility (Figure 4) shows that there is no net change in the charge of this modified transferrin.

*Effect of Carbohydrate Removal on Transferrin Function.* Morgan *et al.* (1967) have shown that removal of sialic acid from transferrin does not impair the function of the molecule. It was of interest, however, to determine whether removal of the other sugars of the two heterosaccharide chains of transferrin would affect its biologic function. Using purified neuraminidase, virtually 100% of the bound sialic acid could be released from transferrin. However, using a jack bean preparation as a source of glycosidases, it was possible to remove only 40–50% of the remaining carbohydrate from the transferrin. This calculation is based on the data of Jamieson (1965) who has found that there are 4 moles of sialic acid, 8 moles of *N*-acetylglucosamine, 4 moles of galactose, and 8 moles of mannose per 90,000 g of transferrin. Why the glycosidases did not remove more of the sugars is not known. When the enzymatically released sugars were subjected to paper chromatography as described in the Methods section, three spots which corresponded in mobility with the standards mannose, galactose, and *N*-acetylglucosamine were detected with the $AgNO_3$–NaOH reagent. Furthermore, the released sugars reacted in the specific color tests for acetylated amino sugar and mannose (Reissig *et al.*, 1955; Dische *et al.*, 1949). These data suggest that the jack bean preparation contained glycosidases which were capable of removing all the component sugars from transferrin, yet for some reason (possibly the presence in the heterosaccharide chain of a particular linkage resistent to hydrolysis) failed to remove 100% of these sugars.

When the carbohydrate-poor transferrin preparations were tested for their biologic activity, the results shown in Table II were obtained. The sialic acid free transferrin gave results identical with the control, confirming the data of Morgan *et al.* (1967). The jack bean glycosidase-treated transferrin preparations which had approximately 39 and 47% of their remaining sugars removed were able to bind to reticulocytes in a normal fashion and to transfer iron into the cells at a rate somewhat greater than the control transferrin. In another experiment using transferrin which had approximately 30% of the sugar removed, the incorporation of radioactive iron into heme was only 70% of the control value.

These results suggest that the two heterosaccharide chains of transferrin are not essential either for the binding of transferrin to reticulocytes or for the transfer of iron into the cell. Further evidence to support this conclusion is shown in Table III. In this experiment, $^{59}Fe$-labeled transferrin was incubated with reticulocytes in the presence of increasing concentrations of one of the purified intact heterosaccharide chains of transferrin obtained from Dr. Graham Jamieson who prepared it by extensive Pronase digestion of the intact glycoprotein followed by treatment with carboxypeptidase B to remove a single lysine residue (Jamieson, 1967). If the carbohydrate chains played a significant role in the interaction of transferrin with reticulocytes, the isolated chains might act as haptene inhibitors, by analogy to the effect of simple sugars and oligosaccharides on the interaction of ABO antibodies with red blood cells (Watkins and Morgan, 1962). Even at high concentrations the isolated heterosaccharide chains had no effect

TABLE II: Effect of the Removal of Carbohydrate on Transferrin Function.

| | $^{59}$Fe Uptake ($\mu$g) | |
|---|---|---|
| | 0 min | 30 min |
| Expt 1 | | |
| Control transferrin | 0.004 | 0.052 |
| Glycosidase-treated transferrin[a] | 0.006 | 0.059 |
| Expt 2 | | |
| Control transferrin | 0.002 | 0.052 |
| Neuraminidase-treated transferrin[b] | 0.002 | 0.050 |
| Glycosidase-treated transferrin[c] | 0.002 | 0.125 |

[a] 47% of carbohydrate other than sialic acid removed. [b] 100% of sialic acid removed. [c] 39% of carbohydrate other than sialic acid removed.

on the ability of transferrin to transfer iron to reticulocytes.

## Discussion

The data presented in this paper demonstrate that chemical modifications of transferrin may result in marked alterations in the biologic properties of the molecule. In each instance the same groups of the protein (that is, the free amino groups of lysine and the N-terminal amino group) were blocked with reagents which varied in size and charge. Regardless of the reagent, the interaction of the transferrin with reticulocytes was more sensitive to the chemical modifications than the ability of the molecule to bind iron, as measured by chromogenic capacity. All of the modifications, except trinitrophenylation, lead to a decreased affinity of transferrin for the physiologic iron-transfer sites on the reticulocyte cell surface. This effect was most marked when the net charge of transferrin was altered. Thus, 25% acetylated and carbamylated transferrin had poorer affinities for iron-transfer sites than 84% amidinated transferrin. The ability of these altered transferrins to donate their iron after binding to the transferrin receptor sites was minimally affected. Another effect seen with all chemical modifications, except for amidination, was an increased binding of transferrin to sites other than iron-transfer sites on the reticulocyte cell surface. The significance of this binding is not clear. It was specific for reticulocyte cell surfaces and, at least in the case of trinitrophenylated transferrin, the affinity constant for binding to these sites was similar to the affinity constant for iron-transfer sites. By comparison, the affinity constant for the nonspecific binding of normal transferrin to both adult red cells and reticulocytes is an order of magnitude greater.[2] One possible explanation for these observations is that modifications of transferrin lead to binding to physiologic receptor sites involved in the transport of other substances. This possibility deserves further exploration.

The alterations in biologic activity which occur following trinitrophenylation of transferrin are extremely complex. As was true of acetylation and carbamylation, an early change was the appearance of transferrin binding to noniron-transfer sites on the reticulocyte cell surface. When more than 75% of the free amino groups of transferrin had reacted to form trinitrophenylated derivatives, there was a progressive decrease in the iron-binding capacity of the molecule; yet iron that was bound could be transferred to reticulocytes at a somewhat enhanced rate. While the apparent binding affinity for this altered transferrin is less than normal, the data in Figure 10 show that the interaction of trinitrophenylated transferrin with reticulocytes does not follow simple Michaelis–Menten kinetics. The upward slope of the curve suggests that as more transferrin is bound to the cell surface, the binding affinity for the molecule increases. Whether this effect is due to transferrin binding to iron-transfer sites or to nonspecific sites cannot be determined from the data. The results of the competition experiments confirm the complexity of the interaction of this modified transferrin with reticulocytes.

Since the kinetic parameters used to calculate the various constants represent a simplification of a complex situation, it is possible that extensively trinitrophenylated transferrin reacts abnormally in several of the steps involved in transferrin binding and iron transfer with the end result being the peculiar kinetic data obtained. Further studies of the binding of transferrin to its physiologic binding sites on the reticulocyte membrane and the mechanism of the subsequent iron release will be required to fully understand this situation.

TABLE III: Effect of Aspartate Heterosaccharide (ASP-CHO) Chains on Transferrin Function.

| Addns to Reaction Mixture (mg/ml) | | Ratio Asp-CHO Chains: Transferrin CHO Chains[a] | $^{59}$Fe Uptake ($\mu$g) |
|---|---|---|---|
| None | | | 0.0152 |
| Asp-CHO chains | 0.01 | 0.72 | 0.0158 |
| | 0.10 | 7.2 | 0.0159 |
| | 1.0 | 72.0 | 0.0162 |

[a] These calculations are based on the data of Jamieson (1965) that each transferrin molecule (mol wt 90,000) has two heterosaccharide chains which weigh 2350 each. The concentration of transferrin in the reaction mixture was 0.24 mg/ml.

[2] S. Kornfeld, unpublished observations.

The assumption has been made thus far that these reagents react only with the free amino group of lysine and the N-terminal amino acid. The evidence for this assumption comes from the work of other investigators. Okuyama and Satake (1960) and Satake *et al.* (1960) demonstrated that trinitrobenzenesulfonic acid under the mild conditions employed in these experiments reacts only with free amino groups. A very slow reaction occurs with free sulfhydryl groups, but none of these is present in transferrin. No reaction occurs with the guanidinium group of arginine, the hydroxyl group of tyrosine or threonine, or the imidazole nitrogen of histidine. Others have confirmed these observations (Haynes *et al.*, 1967). Wofsy and Singer (1963) have presented data that under the conditions of their experiments only the lysine residues of the protein are amidinated. Likewise, Buttkus *et al.* (1965) whose procedures were followed in this work, have shown that at the low levels of acetylation and carbamylation that were obtained in these experiments, essentially no groups other than lysines were involved.

The failure to implicate the two heterosaccharide chains of transferrin in any of the biologic functions tested leaves unresolved the question of what function the heterosaccharide chains have in this glycoprotein. A deficiency of these experiments was the inability to remove completely all of the carbohydrate from the heterosaccharide chains. However, enough was removed that some biologic effect probably would have been manifest if these carbohydrate chains were important determinants of the functions tested. The failure to show haptene inhibition by the purified Asp heterosaccharide chains supports this conclusion.

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## References

Anson, M. L. (1938), *J. Gen. Physiol. 22*, 79.
Awai, M., and Brown, E. B. (1963), *J. Lab. Clin. Med. 61*, 363.
Buttkus, H., Clark, J. R., and Feeney, R. E. (1965), *Biochemistry 4*, 998.
Dische, Z., Shettles, L. B., and Osnos, M. (1949), *Arch. Biochem. 22*, 169.
Fischer, H. (1941), *Org. Syn. 21*, 53.
Fraenkel-Conrat, H. (1957), *Methods Enzymol. 4*, 247.
Habeeb, A. F. S. (1966), *Anal. Biochem. 14*, 328.
Haynes, R., Osuga, D. T., and Feeney, R. E. (1967), *Biochemistry 6*, 541.
Jamieson, G. A. (1965), *J. Biol. Chem. 240*, 2914.
Jamieson, G. A. (1967), *in* Protides of the Biological Fluids, Vol. 14, Peefers, H. Ed., Amsterdam, Elsevier, p 71.
Jandl, J. H., Inman, J. K., Simmons, R. L., Allen, D. W. (1959), *J. Clin. Invest. 38*, 161.
Jandl, J. H., and Katz, J. H. (1963), *J. Clin. Invest. 42*, 314.
Katz, J. H., and Jandl, J. H. (1964), *in* Iron Metabolism, Gross, F., Ed., Berlin, Springer-Verlag, p 112.
Li, Y. T. (1966), *J. Biol. Chem. 241*, 1010.
Lineweaver, H., and Burk, D. (1934), *J. Am. Chem. Soc. 56*, 658.
Morgan, E. H. (1964), *Brit. J. Haemat. 10*, 442.
Morgan, E. H., and Laurell, C. B. (1963), *Brit. J. Haemat. 9*, 471.
Morgan, E. H., Marsaglia, G., Giblett, E. R., and Finch, C. A. (1967), *J. Lab. Clin. Med. 69*, 370.
Nelson, N. (1944), *J. Biol. Chem. 153*, 375.
Okuyama, T., and Satake, K. (1960), *J. Biochem. 47*, 454.
Reissig, J. L., Strominger, J. H., and Leloir, L. F. (1955), *J. Biol. Chem. 217*, 959.
Satake, K., Okuyama, T., Ohashi, M., and Shinoda, T. (1960), *J. Biochem. 47*, 654.
Schade, A. L. (1964), *Farmaco 19*, 185.
Smithies, O. (1955), *Biochem. J. 61*, 629.
Turnbull, A., and Giblett, E. R. (1961), *J. Lab. Clin. Med. 57*, 450.
Wang, A. C., and Sutton, H. E. (1965), *Science 149*, 435.
Warren, L. (1959), *J. Biol. Chem. 234*, 1971.
Watkins, W. M., and Morgan, W. T. J. (1962), *Vox Sanguinis 7*, 129.
Wofsy, L., and Singer, S. J. (1963), *Biochemistry 2*, 104.