Studies on Human Thyroxine-Binding Globulin

VI. THE NATURE OF SLOW THYROXINE-BINDING GLOBULIN

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ABSTRACT A model system utilizing a highly purified partially desialylated thyroxine-(T_4) binding protein (STBG) was studied. STBG was prepared by the same affinity chromatographic method we have reported for preparation of highly purified T_4-binding globulin (TBG). The necessary prerequisite for preparation of STBG was the use of T_4-substituted Sepharose which had been repeatedly exposed to large volumes of serum for purification of TBG. STBG moved more slowly on cellulose acetate electrophoresis than TBG but had the same molecular weight and antigenic determinants as TBG. It bound T_4 with a 1 : 1 molar ratio but its affinity for T_4 was about 10 times less than that of TB. STBG had about one-fourth the sialic acid content of TBG and the electrophoretic mobility of this protein was similar to that of a T_4-binding protein with a mobility slower than that of TBG which has been seen in the electrophoretic patterns of some normal human serums and in serums of patients with hepatic cirrhosis and which does not appear to be an artifact caused by storage and freezing of serum. This fourth slowly migrating T_4-binding region in electrophoretograms of cirrhotic serums is completely abolished by prior incubation with rabbit antiserum to TBG. The in vitro production of partially desialylated TBG from T_4-Sepharose which had been previously exposed to large volumes of serum may be due to adsorption of neuraminidases to the Sepharose either directly from serum or as the result of bacterial contamination. Partial desialylation of TBG in vivo may be an early step in the catabolism of this protein.

INTRODUCTION

Since the identification in human serum of the three major thyroxine (T_4)[1] transport proteins, thyroxine-binding globulin (TBG), albumin, and thyroxine-binding prealbumin (TBPA), several reports have appeared which identified a fourth T_4-binding protein with an electrophoretic mobility slower than that of TBG. Since this protein has appeared infrequently and inconsistently in the sera examined, speculation has arisen concerning its origin and function. Blumberg and Robbins (1) considered it to be an artifact perhaps arising from serums subjected to long periods of freezing and storage. In a later paper (2) these same authors suggested that this electrophoretically slow moving T_4-binding protein might represent a TBG-albumin complex. Thorson, Tauxe, and Taswell (3) proposed "two gel counterparts" of TBG each with a different molecular conformation as an explanation. Premachandra, Perlstein, and Blumenthal (4) observed an electrophoretically slow-moving TBG in serums obtained from some obese persons and argued that this protein might be the result of an in vivo removal of sialic acid groups from TBG.

We have also observed a T_4-binding protein in normal human serum which has an electrophoretic mobility on cellulose acetate which is slower than that of TBG. This slowly moving protein appears only in occasional samples of human serum and accounts for only a very minor portion of the added T_4 radioactivity measured autoradiographically. We have also found two patients with advanced hepatic cirrhosis whose serums contain large amounts of a slowly moving T_4-binding protein. Since analogous electrophoretic changes in other desialylated glycoproteins are well documented (5), one explanation for the occurrence of a slow electrophoretic form of TBG involved a loss of sialic acid groups. TBG has been reported to have a sialic acid content of five (6) to nine (7) residues per molecule. In view of recent evidence (8) that desialylation of glycoproteins plays an important role in their catabolism, these observations may of-

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[1] *Abbreviations used in this paper:* ANS, 1,8-anilinonaphthalene sulfonic acid; NANA, *N*-acetylneuraminic acid; NHS, normal human serum; T_4, thyroxine; TBG, T_4-binding globulin; TBPA, T_4-binding prealbumin; STBG, desialylated T_4-binding globulin.

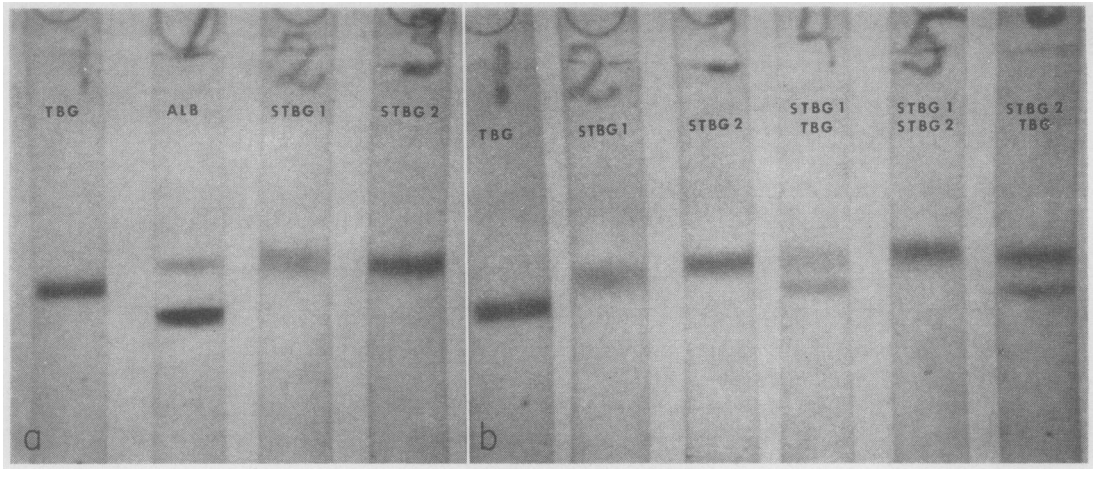

FIGURE 1 (*a*) Disc gel electrophoretogram of highly purified TBG (TBG) and two preparations of slow TBG (STBG 1 and STBG 2). A disc gel electrophoretogram of human serum albumin (ALB) is included for reference. 30–45 μg of each protein was applied to the gels. Gels stained with amido-black. Anode at bottom. (*b*) Disc gel electrophoretogram of TBG, two preparations of slow TBG and combinations of them. Labeled as in Fig. 1*a*. Conditions same as Fig. 1*a*.

fer an explanation for the appearance of the slowly moving T_4-binding protein in the electrophoretic patterns of some human serums.

We have desialylated purified TBG using *Vibrio cholerae* neuraminidase in order to study its physical properties. In addition, we have isolated by affinity chromatography of human serum (9) a highly purified protein which binds T_4, has an electrophoretic mobility on cellulose acetate closely corresponding to that of the slowly moving T_4-binding protein occasionally seen in normal serum, and has the same antigenic determinants as highly purified TBG and TBG in serum. This protein is called STBG. Utilizing this highly purified STBG, we present evidence that it may be analogous to the slowly moving T_4-binding protein often seen in electrophoretic patterns of normal serum.

METHODS

Electrophoresis. Analytic disc gel electrophoresis was carried out by the method of Davis (10) using Tris-glycine buffer, pH 8.2–8.4. The gels were stained with 0.5% amido-black in 7% acetic acid and destained electrophoretically.

Cellulose acetate electrophoresis was carried out in a Beckman Microzone cell (Beckman Instruments, Inc., Fullerton, Calif.) as described previously (11). All samples were labeled with tracer amounts of ^{125}I-labeled T_4 before electrophoresis. After electrophoresis, the cellulose acetate membranes were dried in an oven for 20 min at 90°C. Autoradiography was done by a method previously described (11).

Binding studies. Binding of T_4 to TBG was determined by pressure dialysis in a Lucite cell (Metaloglass, Inc., Boston, Mass.) by a modification of the method of Paulus (12) which we have reported previously (13).

Neuraminidase treatment. Samples of TBG and STBG alone and in combination were treated before electrophoresis with a commercial preparation of *V. cholerae* neuraminidase with a stated activity of 500 U/ml (Behring Diagnostics, Inc., Woodbury, N. Y.). Samples for electrophoresis were prepared as follows: 25 U of neuraminidase and $CaCl_2$ to a final concentration of 0.0015 M was added to approximately 20 μg of protein in sodium acetate buffer, pH 6.2, $\mu = 0.2$. The final volume of the reaction mixture was 0.108 ml and each mixture contained 0.05 μg of T_4-^{125}I. The samples were incubated overnight at room temperature before electrophoresis, as described above. A similar experiment was carried out in which TBG was exposed to *V. cholerae* neuraminidase for timed intervals of from 5 min to 3 hr at 37°C. At the end of each time interval the reaction was stopped by addition of an equal volume of cold 0.2 M Tris-acetate buffer, pH 8.9. All samples were frozen and then thawed immediately before electrophoresis.

Sialic acid determinations. Sialic acid content of TBG, STBG, and bovine mucin substrate was determined by the method of Warren (14), after neuraminidase treatment or mild acid hydrolysis (0.1 N H_2SO_4, 80°C for 1 hr) to liberate sialic acid. A standard curve was constructed using crystalline N-acetylneuraminic acid (Sigma Chemical Co., St. Louis, Mo.). For calculations of N-acetylneuraminic acid residues per mole TBG, a molecular weight of 63,000 was used (13).

Immunologic studies. Immunodiffusion of TBG and STBG against antiserum to TBG was carried out in 1% agar gel. The antiserum to TBG had been raised in New Zealand rabbits as previously described (15). This antiserum was also used in the studies in which normal serum and serums from two patients with advanced cirrhosis of the liver were reacted with TBG antiserum overnight at room temperature and then electrophoresed on cellulose acetate after addition of tracer amounts of radioactive T_4. In this experiment equal volumes of undiluted serum and antiserum were used.

Ultracentrifugation study. The molecular weight of STBG was determined in a Spinco model E ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) under conditions of sedimentation to equilibrium by the

high speed method of Yphantis (16). The ultracentrifugation was carried out in 0.06 M Tris-HCl buffer, pH 8.6. A value of 0.73 was assumed for the partial specific volume.

Fluorescent studies. Characterization of the binding of T_4 to TBG and STBG was carried out by the method of fluorescence quenching which we have previously reported (17). This procedure, using the fluorescent dye 1,8-anilinonaphthalene sulfonic acid (ANS), exploits the fact that a ligand whose absorption spectrum overlaps the fluorescence emission spectrum of its binding protein will quench the intrinsic fluorescence of that protein on binding.

Preparation of TBG and STBG. Highly purified TBG and STBG was prepared by affinity chromatography from pooled human serums which were obtained from volunteers or pooled blood-bank serum (9).

The protein eluted from the T_4-substituted Sepharose was examined in every case by means of autoradiography of the concentrated eluant after cellulose acetate electrophoresis. While the majority of the affinity chromatography runs yielded TBG with an electrophoretic mobility on cellulose acetate identical with that of TBG in normal serum, certain runs yielded a T_4-binding protein (STBG) with an electrophoretic mobility slower than that of TBG in normal serum.

The factors which might be responsible for the appearance of STBG were systematically investigated. It was determined that the age of the serum pool used as starting material did not affect the electrophoretic mobility of the product, nor did the length of time the serum was exposed to T_4-Sepharose. The appearance of STBG always occurred after elution from the T_4-substituted Sepharose and not at later steps in the purification procedure. It became evident that once a particular batch of T_4-Sepharose produced STBG, it would continue to produce it thereafter and that the batches responsible were those which had been reused many times, and had been contacted with large volumes (4–6 liters) of serum. STBG was never produced from freshly made batches of T_4-Sepharose. Furthermore, T_4-Sepharose which produced STBG could again produce TBG after washing with 0.2 M KOH and reequilibrating with 0.1 M $NaHCO_3$.

Since, as described below, the sialic acid content of STBG was about one-fourth that of TBG, it was suspected that a neuraminidase might be attached to batches of T_4-Sepharose which produced STBG. In order to prove this hypothesis, experiments described below were carried out.

RESULTS

The differences in electrophoretic mobility of TBG and two different preparations of STBG in polyacrylamide disc gels is shown in the stained electrophoretograms in Fig. 1*a*. Highly purified TBG had an electrophoretic mobility between that of the albumin monomer and dimer included as a reference, while the STBG had an electrophoretic mobility slightly slower than that of the albumin dimer.

Fig. 1*b* shows stained disc gel electrophoretograms of TBG, two different preparations of STBG, and combinations of the two. When TBG was combined with either preparation of STBG, two stained bands appeared. The most anodal retained the same mobility as TBG alone, and the most cathodal had the same mobility as STBG and moved as a single band with the same mobility as either of the STBG samples alone. A combination of the two STBG preparations moved as a single band in the gel.

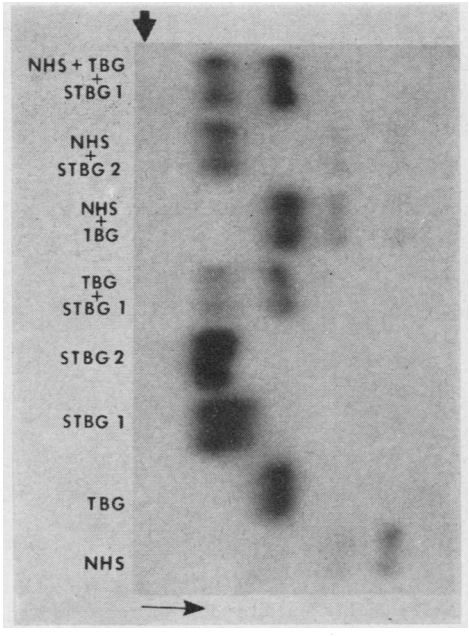

FIGURE 2 Autoradiograph of cellulose acetate electrophoresis of NHS, highly purified TBG (TBG), two preparations of slow TBG (STBG 1 and STBG 2) and various combinations. T_4-^{125}I added to samples before electrophoresis. The horizontal arrow indicates direction of migration; the vertical arrow the origin.

Fig. 2 demonstrates a autoradiograph of a cellulose acetate electrophoresis of various combinations of TBG, STBG, and normal human serum to which tracer amounts of T_4-^{125}I had been added. In this system STBG again migrated more slowly than TBG and both demonstrated T_4 binding. When TBG was added to normal human serum (NHS), intensification of the area of radioactivity associated with TBG in NHS alone occurred. When STBG and TBG were combined and added to NHS the T_4 radioactivity was found in four zones, the most cathodal of which corresponded to that of STBG alone, the second of which corresponded to that of TBG alone, the third to albumin, and the fourth to TBPA. The zones of radioactivity associated with albumin and TBPA (when STBG and TBG were both added to NHS) were present on the original autoradiograph but were very faint because the addition of both STBG and TBG provided a large excess of avid binding sites for thyroxine.

Since the slowly moving T_4-binding protein often observed in NHS had an electrophoretic mobility more closely resembling the mobility of STBG than the mobility of TBG treated with *V. cholerae* neuraminidase (see below), experiments were designed to determine

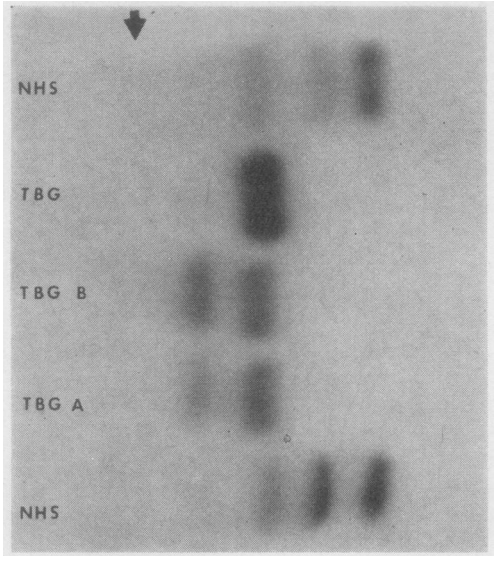

FIGURE 3 Autoradiograph of cellulose acetate electrophoresis of NHS, highly purified TBG after exposure for 20 min to T_4-Sepharose known to produce STBG (TBG A), highly purified TBG exposed overnight to T_4-Sepharose known to produce STBG (TBG B), and highly purified TBG (TBG). Tracer amounts of T_4-^{125}I added to all samples before electrophoresis. Anode to right. The arrow indicates the origin.

whether the STBG produced by some batches of T_4-Sepharose was the result of a neuraminidase associated with the Sepharose.

Highly purified TBG (600 μg/ml) was applied in a volume of 0.25 ml to a 0.9 × 15 cm column packed with T_4-Sepharose known to produce STBG. Elution was carried out as previously described (9) and the eluate concentrated to 0.5 ml by pressure dialysis. After addition of tracer amounts of T_4-^{125}I the concentrated eluate was electrophoresed on cellulose acetate and a autoradiograph made. The above procedure was repeated except that the TBG and T_4-Sepharose were stirred overnight at room temperature before packing in a column and elution. Fig. 3 shows the autoradiograph of this experiment. A significant portion of the TBG exposed to T_4-Sepharose known to produce STBG was converted to STBG and the reaction apparently occurred within the first few minutes of contact with the T_4-Sepharose. Since not all of the TBG was converted to STBG, it is possible that the capacity of the T_4-Sepharose for conversion of TBG to STBG was exceeded by the protein applied to the column.

A second experiment was designed to test the ability of a compound known to block neuraminidase to protect TBG when exposed to T_4-Sepharose producing STBG. Edmond and his coworkers (18) have reported the *N*-substituted oxamic acids as inhibitors of neuraminidase and Cuatrecasas and Illiano (19) have used the inhibitor *p*-aminophenyloxamic acid in their purification of neuraminidase by affinity chromatography. T_4-Sepharose known to produce STBG was equilibrated with *p*-nitrophenyloxamic acid (K & K Labs, Plainview, N. Y.), 20 mg/100 ml in 0.1 M $NaHCO_3$. Highly purified TBG was applied to a column of the inhibitor-treated T_4-Sepharose and eluted as described in the previous experiment. The results of this experiment are shown in the autoradiograph depicted in Fig. 4. The presence of a neuraminidase inhibitor prevented the conversion of TBG to STBG by the T_4-Sepharose. Apparently the inhibitor under the conditions of this experiment did not afford complete protection because the mobility of the eluted TBG is very slightly slower than that of the starting material. This figure also shows that T_4-Sepharose known to produce STBG will completely convert TBG to STBG if the amount of TBG applied to the Sepharose does not exceed its capacity for conversion (cf. Fig. 3). In this experiment less than 25% of the amount of TBG present in the experiment shown in Fig. 3 was exposed to same amount of Sepharose used in Fig. 3. Due to the very small amount of TBG present unbound

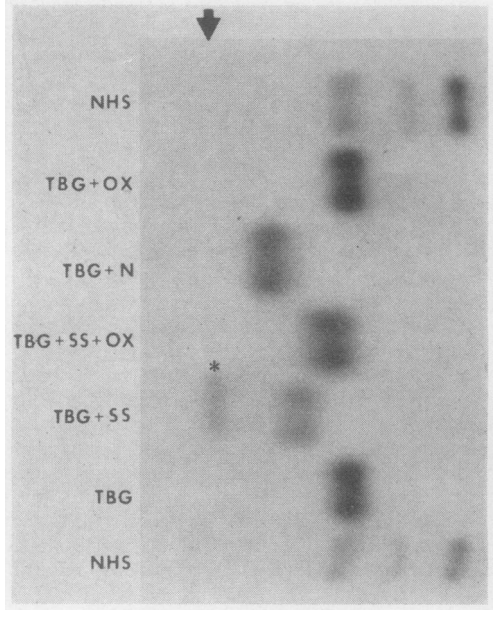

FIGURE 4 Autoradiograph of cellulose acetate electrophoresis of NHS, highly purified TBG (TBG), highly purified TBG after exposure to T_4-Sepharose known to produce STBG (TBG + SS), highly purified TBG after exposure to T_4-Sepharose known to produce STBG which had been previously exposed to *p*-nitrophenyloxamic acid (TBG + SS + OX), highly purified TBG treated with *V. cholerae* neuraminidase (TBG + N), and highly purified TBG exposed to *p*-nitrophenyloxamic acid (TBG + OX). Tracer amounts of T_4-^{125}I added to all samples before electrophoresis. The zone of radioactivity indicated by * is nearly at the origin and is characteristic of the migration of free T_4-^{125}I Anode to right. The arrow indicates the origin.

T_4 activity is seen near the origin (symbol * in Fig. 4).

In the presence of neuraminidase, sialic acid can be released from bovine mucin (20). T_4-Sepharose known to produce STBG was stirred overnight at room temperature with a solution of bovine submaxillary mucin (Sigma Chemical Co.) at a concentration of 5 mg/ml. A volume of mucin equal to the volume of settled Sepharose was used. Free sialic acid was determined by the Warren assay (14). The results indicated that 2 ml of T_4-Sepharose known to produce STBG could liberate about 5 μg of sialic acid from the mucin whereas T_4-Sepharose which produced TBG did not liberate any sialic acid.

The molecular weight of STBG as determined in the ultracentrifuge was 56,500. Molecular weight of STBG determined by the polyacrylamide gel method of Hedrick and Smith (21) was approximately 65,000.

The *N*-acetylneuraminic acid (NANA) contents of TBG and STBG as determined by the method of Warren (14) were significantly different and suggested that STBG had lost approximately three-quarters of its sialic acid. The results of this experiment, presented

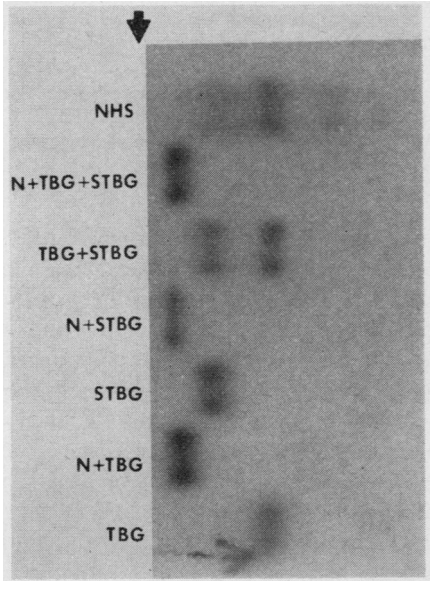

FIGURE 5 Autoradiograph of cellulose acetate electrophoresis of NHS, highly purified TBG (TBG), STBG (STBG), and each of these proteins after overnight incubation with *V. cholerae* neuraminidase (N). See text for details. T_4-^{125}I added to samples before electrophoresis. The horizontal arrow indicates direction of migration; the vertical arrow the origin.

in Table I, indicated that on the basis of a molecular weight of 63,000, TBG contains 9–10 sialic acid residues per molecule and STBG contains about 3 residues per molecule.

To explore the effect of the loss of sialic acid groups from TBG and STBG, these proteins were treated

TABLE I
Sialic Acid Content of TBG and STBG

	NANA	Residues NANA/mole protein*
	%	
TBG	4.86±0.46	9.90
STBG	1.22±0.13	2.48

* Mol wt TBG and STBG 63,000.

with *V. cholerae* neuraminidase. Fig. 5 shows the autoradiograph of a cellulose acetate electrophoresis of NHS and various combinations of STBG and TBG with and without incubation with neuraminidase, all enriched with T_4-^{125}I. When neuraminidase was added to TBG, STBG or a combination of the two, a single zone of radioactivity with an electrophoretic mobility even more cathodal than that of STBG appeared. Fig. 5 also shows a diffuse area of radioactivity more cathodal than TBG in the autoradiograph of NHS. This slowly migrating radioactive zone corresponds closely in its electrophoretic mobility with that of STBG.

The effect of progressive loss of sialic acid groups on electrophoretic mobility of TBG was investigated. Fig. 6 shows the autoradiograph of a cellulose acetate electrophoresis of purified TBG and TBG after exposure to *V. cholerae* neuraminidase for periods of time ranging from 5 to 40 min. There was a progressive decrease in the mobility of TBG up to 40 min, the greatest increment occurring in the first 5 min of exposure. We have carried out this experiment over 3 hr and find little difference in the mobility of TBG after 40 min of incubation with neuraminidase.

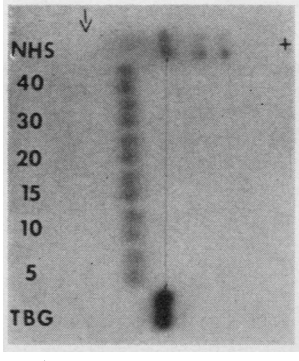

FIGURE 6 Autoradiograph of cellulose acetate electrophoresis of highly purified TBG (TBG) and the same TBG preparation after incubation with *V. cholerae* neuraminidase for the time in minutes indicated by the numbers. Normal human serum (NHS). The anode is indicated by + and the arrow indicates the origin. T_4-^{125}I added to samples before electrophoresis. A line has been drawn between TBG and TBG in NHS for reference.

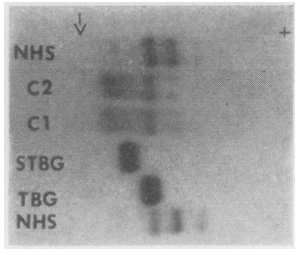

FIGURE 7 Autoradiograph of cellulose acetate electrophoresis of NHS, highly purified TBG (TBG), slow TBG, (STBG), and serum from two persons with advanced cirrhosis of the liver (C 1 and C 2). Anode indicated by + and origin is indicated by arrow. T_4-^{125}I added to samples before electrophoresis.

If the desialylated protein is cleared by the liver as suggested by others (*22*), some patients with hepatic cirrhosis might be expected to have an increased concentration of the desialylated protein in their serum. Fig. 7 shows a autoradiograph of a cellulose acetate electrophoretogram of NHS, TBG, STBG, and serums from two patients with severe cirrhosis and hepatic failure. In the presence of hepatic failure there appeared an electrophoretically slow moving T_4-binding protein which had an even more cathodal position than that of purified STBG, corresponding in its electrophoretic mobility more nearly with that of TBG treated with *V. cholera* neuraminidase. This has been demonstrated in only these 2 cirrhotic serums among the 10 we have tested.

Fig. 8 shows the results of immunodiffusion of TBG, STBG, a combination of equal amounts of TBG and STBG, and NHS alone against rabbit antiserum to TBG. In all cases a single precipitin line formed, indicating antigenic identity of purified TBG, STBG, and TBG in serum.

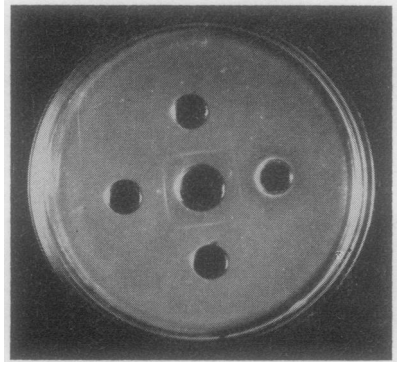

FIGURE 8 Double diffusion in agar. Center well contained rabbit antiserum to highly purified TBG. Top well contained STBG, the right well TBG, the bottom well equal amounts of STBG and TBG, and the left well normal human serum.

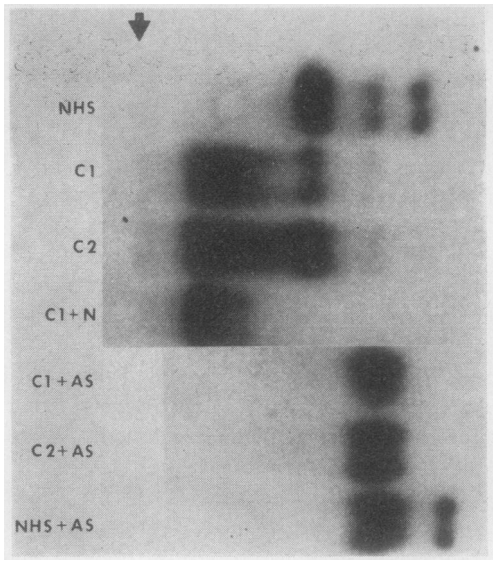

FIGURE 9 Autoradiograph of cellulose acetate electrophoresis of NHS, serum from two patients with advanced hepatic cirrhosis (C 1 and C 2), serum from one of the patients with hepatic cirrhosis after the serum had been treated with *V. cholerae* neuraminidase (C 1 + N), serum from the patients with hepatic cirrhosis after exposure overnight to antiserum to TBG (C 1 + AS and C 2 + AS), and normal human serum after overnight exposure to antiserum to TBG (NHS + AS). Tracer amounts of T_4-^{125}I added to all samples before electrophoresis. Anode to right. The arrow indicates the origin.

Equal volumes of undiluted serum and TBG antiserum were combined and incubated overnight. The combination was subjected to electrophoresis on cellulose acetate after addition of tracer amounts of radiothyroxine and the resulting electrophoretogram autoradiographed. The results are shown in Fig. 9. In the case of normal serum addition of TBG antibody resulted in disappearance of the TBG radioactivity. When serum from the two patients with advanced hepatic cirrhosis was treated with TBG antiserum, both the area of radioactivity associated with TBG and the more diffuse slowly moving area of radioactivity disappeared. The area of radioactivity corresponding to albumin was broader in those samples in which TBG antiserum was present reflecting the superimposition of rabbit albumin on human albumin. Fig. 9 also shows the result of treating serum from one of the patients with hepatic cirrhosis with *V. cholerae* neuraminidase. The radioactivity associated with TBG was eliminated while the diffuse slowly migrating area cathodal to TBG became more compact and more sharply defined.

The binding of T_4 to TBG and to STBG was also demonstrated by the method of fluorescence quenching. Titration of a solution of TBG (1.41 μM) or STBG (1.05 μM) in 0.05 M Tris-0.10 M NaCl, pH 7.3, at 23°C,

with small portions of T_4, produced the curves shown in Fig. 10. Both TBG and STBG bound the added T_4 as shown by the decrease in fluorescence resulting from addition of T_4. The existence of a plateau beyond which the addition of further T_4 failed to yield further quenching indicated saturation of the protein-binding sites. Extrapolation of the initial slope of the quenching curve —representing essentially complete binding of added ligand—described a line representing mole of *bound* T_4 per mole of TBG vs. relative fluorescence. The intersection of this line with the plateau yielded the stoichiometry of the interaction: i.e., moles of ANS bound at saturation of TBG. The initial slope was calculated by the method of least squares. Fig. 10 indicates that 0.85±0.06 moles of T_4 bound per mole of TBG and 0.91±0.05 bound per mole of STBG. Thus, both the native and the desialylated protein had the same binding capacity.

Fig. 10 also shows that the extent to which TBG intrinsic fluorescence is quenched by T_4 at saturation (25%) differs from the degree to which STBG is quenched (50%). Quenching efficiency has been shown to depend upon the relative orientation of the acceptor and donor dipoles and upon the distance between the two species (23).[2]

Preliminary experiments utilizing the ultrafiltration cell (12) to determine the equilibrium constant of STBG have shown at least a 10-fold decrease in the kEq of STBG as compared with the previously determined (17) kEq of 2.35×10^{10} M^{-1} for TBG.

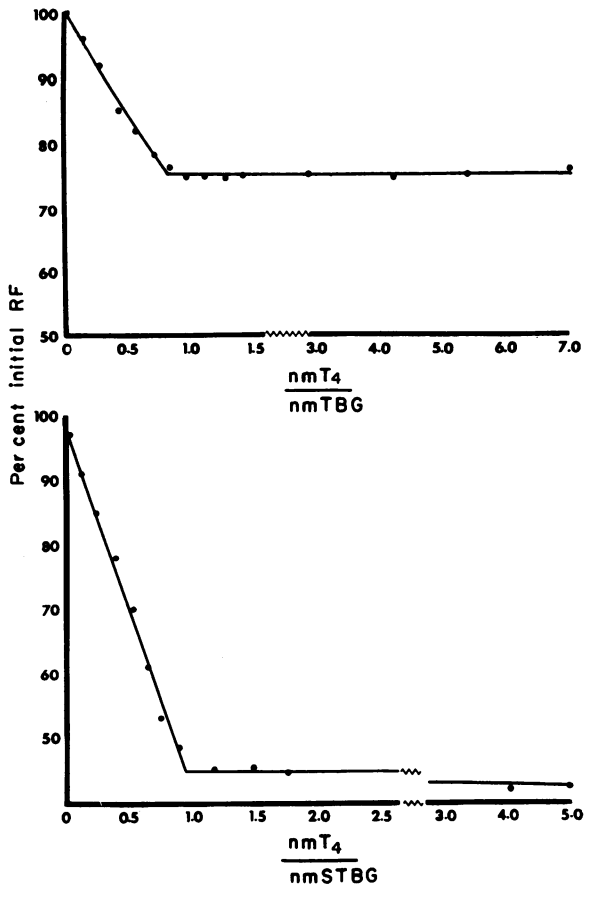

FIGURE 10 *Top.* Plot of relative fluorescence at 475 nm vs. molar ratio of T_4 to TBG. Portions of T_4 were added to a solution of TBG (1.11×10^{-6} M) and ANS (1.0×10^{-5} M) in 0.05 M Tris, 0.10 M NaCl (pH 7.3). *Bottom.* Same as above except the plot is of relative fluorescence at 475 nm vs. ratio of T_4 to STBG. In both cases excitation was 400 nm and emission 475 nm.

DISCUSSION

Accumulation of recent evidence suggests that the inclusion of carbohydrate residues in the structure of many proteins may be an important means of coding the protein for its eventual location within the organism possibly to provide a means of recognition of the protein for its target cell (25). As an extension of this hypothesis there is evidence for certain glycoproteins that the recognition code may be hidden by terminal sialic acid residues and thus does not operate until removal of these residues at which time the protein may become recognized by hepatic cells as ready for catabolism (8, 22). The availability of a purified partially desialylated TBG (STBG) provided a new model system in which a serum glycoprotein and its partially desialylated congener could be closely studied. In addition, observations on the properties of STBG provided evidence for an explanation of the fourth T_4-binding protein seen in certain normal and pathologic human serums.

The elusive fourth T_4-binding protein has been present in many but not all serums we have examined (see Fig. 5) and has been previously observed by other investigators (1–4). This slow moving band does not necessarily appear to be an artifact arising from storage and freezing since we have observed it in serums which had been subjected to electrophoresis within an hour of venipuncture; further, it does not appear more frequently in frozen serums as compared with unfrozen samples. Supporting evidence that the slower moving

[2] Assuming constant geometry between donor aromatic residues and the acceptor ligand, the efficiency of quenching is related to inter-species distance as: $E = \frac{1}{(1 - (r/r^0)^6)}$ (24). Assuming the tryptophan-T_4 critical distance (r^0) is 15.4 A, r for TBG is 20.8 A and for STBG is 15.0 A. Thus, assuming constant geometry between donor and acceptor, removal of sialic acid residues appears to decrease the distance between bound T_4 and the protein aromatic residues by about 6 A. However, an effect on the dipole orientations secondary to removal of the charged groups obviously cannot be ruled out as contributing to the apparent distance change calculated by this method.

T_4-binding protein is, in fact, a form of TBG is provided by the observation that preincubation of serums from two patients with advanced hepatic cirrhosis with anti-TBG globulin causes the slowely moving area of T_4 binding to disappear as well as that associated with TBG. The diffuse nature of the slowly moving T_4 band in these serums suggests that there may be multiple entities binding T_4 and interacting with anti-TBG. Finally, treatment of these cirrhotic serums with neuraminidase causes the TBG band to disappear and the slowly migrating diffuse area to become more sharply defined at its cathodal edge. While other explanations of these phenomena are not ruled out, these observations can be consistently and simply explained by postulating the slow moving T_4-binding band to be composed of a microheterogeneous TBG which has been desialylated to varying degrees.

The conversion of a significant portion of TBG to STBG when exposed to T_4-Sepharose which had formerly produced STBG, the lack of conversion of TBG to STBG when exposed to the same T_4-Sepharose which had been equilibrated with a neuraminidase inhibitor, and the demonstration of liberation of free sialic acid from mucin by this T_4-Sepharose preparation and not by T_4-Sepharose producing TBG argues for the action of a neuraminidase or a substance with neuraminidase-like activity. The presence of neuraminidase on T_4-Sepharose which had been exposed to large volumes of human serum could be accounted for by nonspecific adsorption of endogenous neuraminidase from serum or could be the result of neuraminidase derived from bacterial contaminants. Preliminary experiments have been carried out in which T_4-Sepharose known to produce STBG has been cultured in broth after which TBG has been exposed to the culture broth. Subsequent cellulose acetate electrophoresis showed that the culture broth did not convert TBG to STBG. This argues against bacterial contamination as the source of the neuraminidase-like activity. As yet we have not carried out experiments to eliminate the possibility of viral contamination. The nature of this neuraminidase is currently under investigation.

The evidence indicates that STBG is similar to TBG in its antigenic determinants, its molecular weight and its capacity for binding T_4. However, STBG has a slower electrophoretic mobility on cellulose acetate and in polyacrylamide gels than TBG and contains only one-fourth as much sialic acid. The binding constant of STBG for T_4 appears to be at least 10-fold less than that of TBG. The data on quenching of protein fluorescence of STBG and TBG by T_4 are consistent with the formation of a less extended molecule upon desialylation leading to a partial restriction or hindrance of the binding site. While the loss of sialic acid residues with their repelling static charges may indeed affect molecular configuration or hydration, the fluorescence differences may also reflect such factors as a change in the distribution of quenching groups.

Van den Hamer, Morell, Scheinberg, Hickman, and Ashwell (22) have observed that different neuraminidase preparations had preferential affinity for different sialic acid residues on ceruloplasmin and that removal of any two of the sialic acid residues hastened the disappearance of the protein from the circulation of rats. The protein that was rapidly cleared could be recovered from the liver. Morell, Gregoriadis, Scheinberg, Hickman, and Ashwell (8) have extended these observations to include a number of other glycoproteins. Only desialylated transferrin showed a survival time in the circulation that was the same as the native protein. These investigators postulated that removal of sialic acid residues from glycoproteins permitted recognition of the "defective" molecule by hepatic cells and that this constituted a normal mechanism for the catabolism of proteins. This hypothesis is supported by our observation of a slow-moving T_4-binding protein antigenically similar to TBG in cirrhotic serums. Massive hepatic parenchymal destruction would be expected to interfere with the clearance of "defective" protein from the circulation. This decreased clearance would, of course, be accompanied by decreased serum protein synthesis by the liver, and not all cirrhotics would demonstrate this finding.

We have carried out preliminary experiments in which TBG and STBG have been radioiodinated and injected intravenously in tracer amounts into rats. The results suggested that STBG initially disappeared from the circulation at about twice the rate of TBG and that STBG radioactivity appeared in the liver more rapidly and in greater quantity than that of TBG. Further experiments on the catabolism of TBG and STBG are in progress and will be reported elsewhere.

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