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Determination of Terminal Sugars in Transferrin by Radio-lectin Immunoassay (RLIA)—A New Microanalytical Procedure

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ABSTRACT

A new method for the determination of terminal sugars in immunologically defined glycoproteins with microheterogeneity in the sugar residue has been developed. The method has been elaborated for transferrin and involves the following three steps: 1. Binding of antitransferrin antibodies to cyanogen bromide activated Sepharose. 2. Adsorption to the antitransferrin gel of transferrin from serum or from standard solutions of defined composition of terminal glycoprotein sugars. 3. Adsorption of a ^{125}I - labelled lectin to the Sepharose-antitransferrin-transferrin complex. A galactose-binding lectin from *Crotalaria juncea* and a sialic acid-binding lectin from *Limulus polyphemus* have been successfully labelled with the Bolton-Hunter reagent and used in the radio-lectin immunoassay determinations. Care must be taken that significant amounts of lectins are not lost from the transferrin complex during washing. The resolving power of the method was at best 20 pmoles of asialotransferrin, a figure that probably can be improved significantly by optimizing the assay conditions.

INTRODUCTION

Various biochemical abnormalities are often encountered in clinical practice in association with alcoholism. Some of these are commonly used markers in the diagnosis of alcoholism, as for example, increased gamma-glutamyltransferase activity in serum (8,12,13) increases in serum alpha-lipoproteins (6,7) and elevated serum iron values (21,22). The disadvantage of these and other tests is, however, their lack of specificity for alcoholism.

A new and very specific marker for alcoholism has recently been described, which so far has shown a high specificity for prolonged, high alcohol consumption (18,19,20). Serum transferrin of alcoholic patients displays a selective and often marked increase in one of its minor cathodal components when examined by isoelectric focusing. This component has an isoelectric pH (pI) of 5.7, whereas the normal main component has a pI of 5.4. The difference in

pI is most probably due to the different content of sialic acid (10,15,18).

The present laboratory routines for demonstrating this transferrin abnormality involves isoelectric focusing followed by immunofixation and subsequent quantitative densitometry. This is a time-consuming procedure, which requires special equipment (20). Thus, the method does not readily lend itself to clinical use on a larger scale, and a simpler method of assessing terminal carbohydrates in transferrin was required. The present report describes a method, which besides being simpler also presents a new, extremely sensitive general method of assessing terminal carbohydrates in glycoproteins. Since there are more clinical conditions characterized by plasma glycoconjugate patterns, we think that this method may find wider application in the future. The name radio-lectin immunoassay, abbreviated RLIA, is proposed.

METHODS

Binding of antitransferrin to Sepharose 4 B.

Rabbit anti-human transferrin from Dakopatts, Copenhagen, Denmark (2mg/ml) was concentrated to 10 mg/ml by vacuum dialysis and the solvent exchanged by dialysis to sodium phosphate buffer (0.07 moles/1, pH 8.0). The solution was transferred to room temperature prior to cyanogen bromide activation of Sepharose.

The Sepharose-gel (Pharmacia AB, Uppsala, Sweden) was activated with cyanogen bromide as follows (cf. 1): 30 ml of Sepharose 4 B were washed with redistilled water, chilled to 20°C and transferred to a beaker with magnetic stirring. 5 g of cyanogen bromide was added and the pH was maintained at 11 by addition of sodium hydroxide from an ice-cold solution of 2 moles/1. The temperature was kept at 20°C by addition of crushed ice. When the titration was almost complete (usually after 15 min), the gel was transferred to an ice-cold Büchner funnel (100 ml) and washed with portions of ice-cold sodium phosphate buffer (1.5-2 1 of 0.07 moles/1 of phosphate, pH 8.0). An amount of gel equal to that of the antitransferrin solution was transferred to the latter and allowed to stand at room temperature with magnetic stirring for 2 h. Ethanolamine-HCl (1 mole/1, pH 8.0) was then added (5 ml per ml of gel) and the mixture incubated with stirring for an additional 2 h (5). Finally, the buffer was exchanged by washing and centrifugation to 0.05 moles/1 of sodium phosphate buffer, pH 7.5 containing 1 mole/1 of sodium chloride. The gel was stored at 4°C in an equal volume of this buffer.

Characterization of the transferrin-binding properties of the antitransferrin gel.

The transferrin-binding properties of the gel were analyzed by recording the binding of ^{125}I - labelled transferrin (see below), and by incubation with

human serum followed by elution of bound serum proteins at acid pH, and subsequent determination of the transferrin content by immunodiffusion, or by characterization of the bound serum proteins by isoelectric focusing.

To determine the transferrin-binding properties of the gel by the affinity of ^{125}I-transferrin, 10 µl of gel were incubated with 4-100 µg of transferrin (1g/l in phosphate-buffered saline, pH 7.4) containing ^{125}I-transferrin as a tracer for 10 min at 37°C in Ellerman plastic centrifuge tubes (volume 2 ml), followed by dilution, pelleting and washing. The bound ^{125}I-transferrin was assessed by liquid scintillation in a Nuclear Chicago Unilux II equipment.

For the study of binding of serum proteins, 2.5 ml of serum was diluted with 2.5 ml of sodium phosphate (0.05 moles/l)-citrate (0.02 moles/l), pH 7.2 and passed at 5 ml/h through a column containing 1 ml of antitransferrin-Sepharose. (cf. 2). The gel column was thereafter washed with 5 ml of sodium phosphate (0.025 moles/l)-citrate (0.01 moles/l), pH 7.2 and 5 ml of sodium phosphate (0.07 moles/l), pH 7.0 and the bound proteins eluted at 5 ml/h with 5 ml of sodium phosphate (0.025 moles/l)-citrate (0.01 moles/l), pH 2.8. The eluate was neutralized with a solution of saturated Na_2HPO_4 and the buffer exchanged by dialysis to phosphate-buffered saline, pH 7.4.

After affinity chromatography and pH adjustment, the eluted transferrin was incubated at 37°C for 1 h with $FeCl_3$ to a final concentration of 0.2 mmoles/l (11). Thereafter, the transferrin pattern was analyzed by isoelectric focusing in polyacrylamide gel (18). The concentration of transferrin was determined by single radial immunodiffusion according to Mancini et al (9) on M-Partigen Immunodiffusion plates (Behring, Marburg, W. Germany). 28 µg of transferrin from the eluted fraction was subjected to analytical isoelectric focusing in polyacrylamide gel (T=6%, C=3%) in a pH gradient from pH 2.2 to 11.0 (18,23,24). The gel was stained with Coomassie Brilliant Blue R 250 (ICI, Manchester, England) (23). The protein pattern in the eluate was compared with that of immunofixed serum transferrin from alcoholic patients and controls (16,18,20).

Quantitative immunofixation following isoelectric focusing of serum transferrin.

The transferrin concentration in the serum samples from control persons and alcoholic patients was determined by single radial immunodiffusion (9) on M-Partigen Immunodiffusion Plates (Behring, Marburg, W. Germany). Samples containing 0.5-0.8 µg of transferrin were subjected to analytical thin-layer isoelectric focusing in polyacrylamide gel (T=6%, C=3%) in a pH gradient from pH 2.2-11.0 (18,20). The transferrin was immediately immunofixed in the gel with monospecific antitransferrin antibodies (Dakopatts,

Copenhagen, Denmark). The gel was thereafter thoroughly washed and stained with Coomassie Brilliant Blue R 250 (16,18,20). The stained, immunofixed transferrin was quantified by computerized densitometry using a Vitatron TLD densitometer (Dieren, Holland). The amount of the cathodal transferrin component with pI 5.7 was expressed as a percentage of the total immunofixed transferrin (20). The variation of this method is 0.9% for the calculated quotient and the relationship between the densitometric peak area and the applied transferrin amount is linear provided that antibody excess prevails (20).

Serum samples

The control subjects were all considered healthy on the basis of medical history and physical examination. In no instance was any regular heavy alcohol consumption suspected. The alcoholic patients were treated at the Clinical Department of Alcohol and Drug Research, Karolinska Hospital, Stockholm and all fulfilled the criteria of dependence according to WHO (25). The admitted average consumption was in all cases more than 60 g of ethanol/day.

Serum samples were drawn in the morning from fasting subjects. In the case of alcoholic patients, the samples were taken on the first day following admittance to the hospital. The samples were frozen immediately at -25°C and stored for at most 6 months before analysis (17).

Labelling of *Crotalaria juncea* galactose-binding lectin and other proteins with Bolton-Hunter reagent.

The Crotalaria lectin (3,4) which was a generous gift from Dr. Bo Ersson, Uppsala was solubilized in sodium phosphate buffer (0.07 moles/l, pH 8.0) to a concentration of 10 g/l, and the solution chilled on ice. 0.09 nmoles of monoiodinated ^{125}I-Bolton-Hunter reagent (2200 Ci/mmole, NEN Chemicals, GmbH, Frankfurt-on-Main, Germany) in 20 µl of anhydrous benzene were concentrated under a stream of nitrogen in a glass centrifuge tube (10 ml). Approximately half the volume of benzene was evaporated at room temperature (10 min) and the other half evaporated after the tube had been put in an ice-bath (20 min). When the benzene had been evaporated, 50 µg of Crotalaria lectin in 5 µl of sodium phosphate buffer (0.07 moles/l, pH 8.0) with or without addition of 50 mmoles/l of galactose were added from an ice-cold micropipette and allowed to stand for 1 h at 0°C and then for 3 h at 4°C. Subsequently, 1 mg of Crotalaria lectin from the above-mentioned solution was added and the macromolecular material eluted in the void volume from a Sephadex G-25 column (PD-10, Pharmacia, Uppsala, Sweden) with simultaneous buffer exchange to sodium phosphate (0.05 moles/l, pH 7.5) containing sodium chloride (1 mole/l). Fractions of 1 ml were collected and the peak of radioactivity collected in 2 ml. The lectin remained active for at least 4 months when stored at 4°C in the latter buffer.

Galactose-binding was assessed by the affinity of the lectin to partially hydrolyzed Sepharose 4 B (see below).

Besides Crotalaria lectin, human serum transferrin (AB Kabi, Stockholm, Sweden) and antitransferrin (Dakopatts, Copenhagen, Denmark) were labelled as described above. The lectin from *Limulus polyphemus* (Sigma, S:t Louis, Mo.) was labelled at a lower concentration (1 g/l), and at a ratio of protein to Bolton-Hunter reagent of one tenth of that described above. After labelling, the latter lectin was diluted with an equal volume of sodium ethylene-dinitrilo-tetra-acetate (Na-EDTA, 0.1 moles/l, pH 7.4) and transferred to a buffer composed of Tris-HCl (0.05 moles/l, pH 7.2), sodium chloride (0.15 moles/l) and calcium chloride (0.01 moles/l).

Partial hydrolysis of Sepharose 4 B.

A method for partial hydrolysis of Sepharose 6 B previously described by Ersson (4) was used. 50 ml of Sepharose 4 B in a total volume of 150 ml of hydrochloric acid (0.20 moles/l) where shaken in a water bath at 50°C and then transferred to sodium acetate buffer (0.05 moles/l, pH 6.0) containing sodium chloride (1 mole/l). 10 µl of gel in a total volume of 20 µl of this buffer was used to assess the binding of Crotalaria lectin.

Digestion of transferrin with agarose-bound neuraminidase.

0.069 U of agarose-bound neuraminidase from *Dactylium dendroides* (Sigma), with an activity of 44 U/g agarose (1 U liberates 1 µmole/min of sialic acid from AcNeu-lactose at pH 5.0 and 37°C) were transferred to sodium phosphate buffer (0.01 moles/l, pH 6.3). 1 mg of human transferrin (from Sigma, S:t Louis, Mo. or from AB Kabi, Stockholm, Sweden)was dissolved in this buffer, and the solution added to the agarose to a total volume of 1 ml and incubated with shaking for 30 min at 37°C. The agarose was pelleted at the bottom of the tube and asialotransferrin transferred to another tube. 1 mg of untreated transferrin was dissolved in the same buffer and mixtures of the untreated and neuraminidase-digested transferrin prepared, giving various percentages of asialotransferrin and transferrin.

Determination of terminal sugars in transferrin by radio-lectin immunoassay.

10 µl of antitransferrin-Sepharose gel in a total volume of 20 µl of sodium phosphate buffer (0.05 moles/l, pH 7.5) containing sodium chloride (1 mole/l) were pipetted into Ellerman plastic centrifuge tubes cooled in ice. 50 µg of transferrin-asialotransferrin in 50 µl of phosphate buffer (0.01 moles/l, pH 6.3) were then added and the tubes capped and incubated with shaking at 37°C for 10 min. Alternatively, 0.1 ml of sodium phosphate (0.05 moles/l) - citrate (0.02 moles/l), pH 7.2 were added followed by 0.1 ml of serum and incubation

at 37°C for 10 min.

The gel was thereafter washed free of soluble transferrin and incubated with 50-100 µg of ^{125}I-labelled Crotalaria lectin for 10 min in a thermostated shaker at 37°C. The pelleted gel was washed 3-8 times at room temperature in 0.5 - 2 ml of the sodium phosphate-chloride buffer of pH 7.5 described above and finally suspended in 0.5 ml of buffer. This solution was transferred to a scintillation vial and counted in the presence of Aquasol (NEN Chemicals).

When terminal sugars were analyzed by the affinity of the sialic acid - binding lectin from *Limulus polyphemus* to the Sepharose-antitransferrin-transferrin complex, 0.1 ml of Na-EDTA (0.1 moles/l, pH 7.4) was added to the latter and the buffer exchanged by washing with Tris HCl (0.05 moles/l, pH 7.2) containing sodium chloride (0.15 moles/l) and calcium chloride (0.1 moles/l). Approximately 20-25 µg of the Limulus lectin dissolved in 0.1 ml of the latter buffer were then added, followed by three washes.

RESULTS

Binding of transferrin to solid phase.

Antitransferrin (0.2 mg) was either equilibrated with 10 µl of Protein A -Sepharose (Pharmacia AB, Uppsala, Sweden), followed by pelleting and aspiration of excess antitransferrin, or bound directly to cyanogen broide -activated Speharose as described previously. The transferrin-binding capacity of the two types of gel was assessed by equilibration with ^{125}I-labelled transferrin or by immunodiffusion. Protein A - Sepharose - antitransferrin gel bound approximately 10 g/l of transferrin as judged from the saturating concentration of transferrin in the presence of ^{125}I-labelled transferrin added as a tracer (Fig. 1 A). The binding capacity of antitransferrin-Sepharose was 5 g/l using this method (Fig. 1 B). The release of bound transferrin during washing in sodium phosphate (0.05 moles/l) - sodium chloride (1 mole/l) buffer of pH 7.5 was not recorded. The efficiency of binding of ^{125}I-transferrin was about 10 $\pm$ 5% of the added amount at subsaturating concentrations with either type of gel. Antitransferrin covalently bound to Sepharose was used in the subsequent radiolectin immunoassay determinations. Three batches were prepared and characterized by their binding of ^{125}I-transferrin. The latter gel, containing covalently bound transferrin was also characterized by saturation with serum, followed by elution of the bound serum proteins at low pH, determination of the amount of bound transferrin by immunodiffusion and isoelectric focusing of the eluted serum proteins. The gel bound 3.3 $\pm$ 0.1 mg of transferrin per ml of gel as judged from immunodiffusion of a sample from the eluate obtained using the procedure referred to under "Methods". (4 expts.). In one experiment, the serum-phosphate-citrate mixture was recycled through the affinity gel for 2 h giving a figure of 4.25 mg of bound transferrin per ml of gel. The gel pattern after

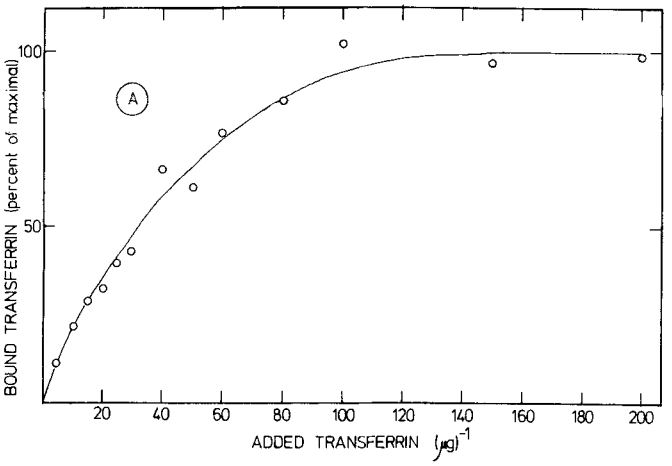

Fig. 1 A. The binding of transferrin to 10 µl of Protein A - activated Sepharose gel (Pharmacia AB, Uppsala) equilibrated with 0.2 mg of transferrin antibodies (Dakopatts, Copenhagen).

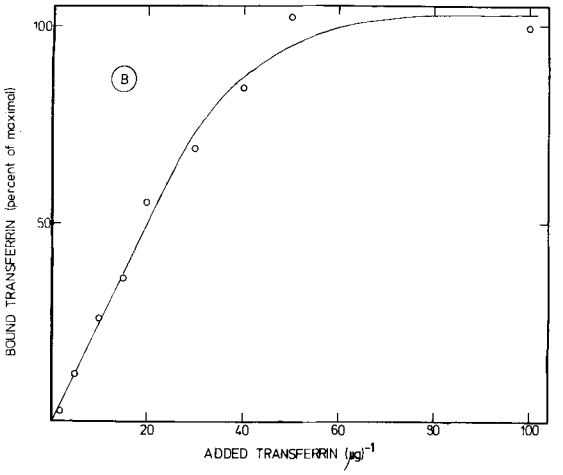

Fig. 1 B. The binding of transferrin to 10 µl of Sepharose 4 B containing covalently bound transferrin antibodies.

isoelectric focusing of the serum proteins having affinity for the antitransferrin gel is shown in Fig. 2 A.

The gel pattern after isoelectric focusing of transferrin from an alcoholic subject is shown in Fig. 2 B and that from a control patient in Fig. 2 C. The transferrin-containing bands in Fig. 2 B and Fig. 2 C were developed by direct immunofixation with antitransferrin antibodies followed by staining with

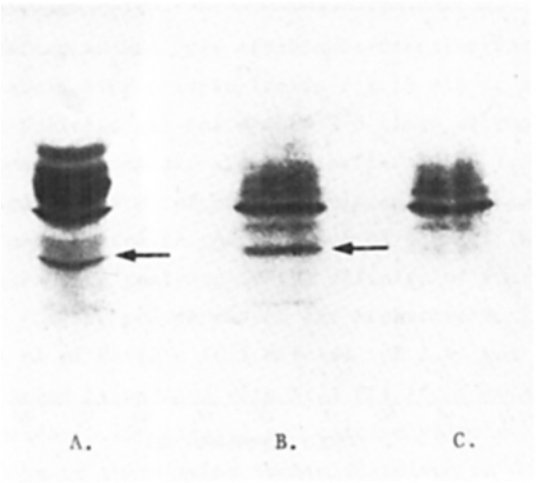

Fig. 2. Analytical thin-layer isoelectric focusing in polyacrylamide gel in a pH gradient pH 2.2-11.0. The anode is at the top. Only the anodal half of the gel is shown. A: Conventional staining of the eluted and iron saturated fraction after affinity chromatography of serum transferrin from an alcoholic patient. The sample contained 28 µg of transferrin. B: Isoelectric focusing followed by direct immunofixation with monospecific antitransferrin antibodies of a serum sample from an alcoholic patient. The sample contained 0.8 µg of transferrin C: Same as in B but from a control person showing the normal transferrin pattern.
The desialotransferrin component with pI 5.7 is indicated by arrows.

Coomassie Brilliant Blue (20). The desialotransferrin component associated with excessive alcohol consumption which is indicated by an arrow in Fig. 2 A and 2 B has an isoelectric point of 5.7 whereas fully sialylated transferrin has an isoelectric point of 5.4. The desialotransferrin component of pI 5.7 occurs at low concentrations (3.7 $\pm$ 1.5 per cent (S.D.) of the total transferrin) in sera of normal subjects (20) and emerges in large amounts after partial digestion of fully sialylated transferrin with neuraminidase. Partial neuraminidase digestion of transferrin also results in the appearence of transferrin components at pI 5.6 and 5.8. Fully desialylated transferrin has a pI of 5.9. These five bands of transferrin identified by immunofixation with antitransferrin antibodies most probably correspond to the four sialic acid residues of native transferrin and their successive removal by hydrolotic cleavage (cf. 10,15).

Preparation of ^{125}I-labelled Crotalaria lectin and evaluation of its galactose binding properties.

After reacting Crotalaria lectin and Bolton-Hunter reagent as described previously, approximately half the radioactivity was eluted in the void volume from a Sephadex G-25 column. When ^{125}I-labelled Crotalaria lectin was equilibrated with partially hydrolyzed Sepharaose 4 B and the unbound lectin removed, binding was observed even after excessive washing with cold phosphate-sodium chloride buffer. Saturation of lectin binding and inhibition of binding by lac-

tose (10 mmoles/l) in the washing buffer was observed under these conditions. (Fig. 3). The functional properties after radio-labelling with Crotalaria lectin were evaluated by these criteria. Four batches were prepared, with a binding capacity of 10 ± 5% of the added radioiodinated lectin under subsaturating conditions. Successful labelling was observed after interruption of the labelling by diluting with unlabelled lectin after 1 h and 3 h of exposure to Bolton-Hunter reagent, and after interruption with ethanolamine-HCl (1 mole/l, pH 8.0). Similar galactose-binding properties of ^{125}I-lectin were observed after labelling in the presence or absence of galactose (50 mmoles/l).

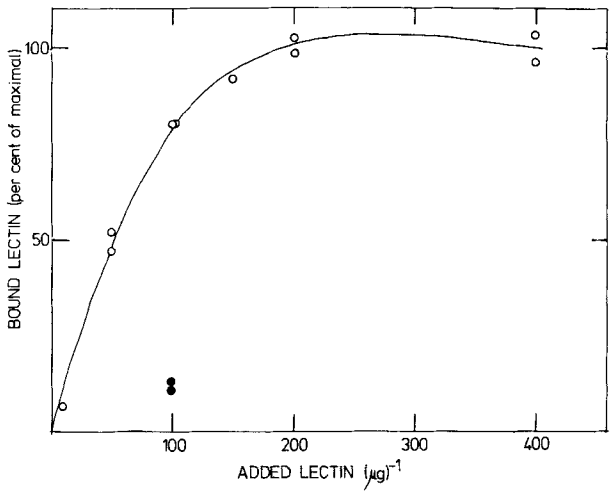

Fig. 3. Verification of galactose-binding properties of ^{125}I-labelled *Crotalaria juncea* lectin to partially hydrolyzed Sepharose 4 B. 10 µl of gel were incubated with the indicated amounts of Crotalaria lectin with labelled lectin added as a tracer. Thereafter, the excess lectin was removed by washing in the absence (empty circles) or in the presence of 10 mmoles/l of lactose (filled circles).

Determination of the terminal sugars in transferrin by radio-lectin immunoassay (RLIA) using lectins from *Crotalaria juncea* and *Limulus polyphemus*.

A molar ratio of transferrin to Crotalaria lectin in the binding assay of 1:1 was chosen. This was based on the assumption that, for steric reasons, maximally one lectin molecule could bind to any of the four (14) galactose residues exposed in an asialotransferrin molecule fixed in the antitransferrin gel. Thus, 10 µl of gel, binding 50 µg of transferrin (MW 60000) were exposed to 50-100 µg of Crotalaria lectin (MW 120000, ref. 4), washed, and the binding estimated. The result of one such galactose assay in which mixtures of transferrin and asialotransferrin were employed, is shown in Fig. 4 A. A characteristic feature of this standard curve is the insignificant lectin binding of

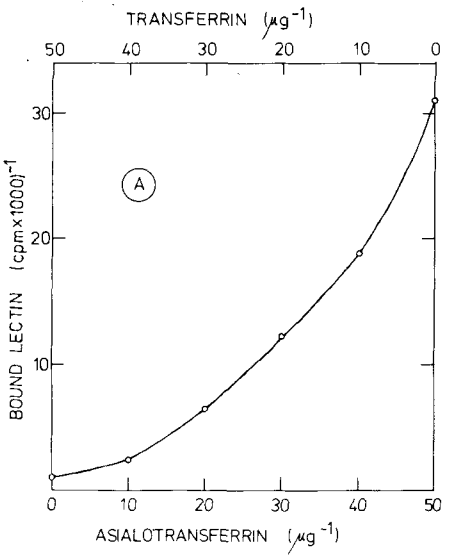

Fig. 4 A. Radio-lectin immunoassay of terminal galactose in transferrin using ^{125}I-labelled Crotalaria lectin. Standard solutions of transferrin were prepared by mixing native transferrin and completely desialylated transferrin. 50 μg of transferrin containing the indicated amounts of the sialylated and completely desialylated form were incubated with 10 μl of antitransferrin-Sepharose gel, the gel washed and subsequently incubated with 50 μg of Crotalaria lectin. After 8 washings, the gel was suspended in 0.5 ml of buffer and the bound lectin measured by liquid scintillation (one determination).

maximally sialylated transferrin and the non-linear gradual increase in lectin-binding as the asialotransferrin/transferrin quotient rises. In the experiment illustrated in Fig. 4 A, the maximal binding observed, corresponds to about 5% of the galactose-binding lectin. This low percentage is due to the fact that significant amounts of lectin are lost from the gel during washing. As shown in Fig. 4 B, the losses of radioactivity from the gels were nearly proportional to the final residues of gel-associated lectin, which is to be expected if galactose-associated lectin is dissolved during washing. 15-20% of the bound lectin were lost in each wash.

The non-linearity of the standard curve illustrated in Fig. 4 A can be explained on the basis of dissociation of lectin from the transferrin-antitransferrin complex. Therefore, the binding assay was repeated within the clinically interesting range of 0-15% asialotransferrin (Fig. 5) but with fewer washings. As can be seen in Fig. 5, for terminal galactose, the slope of the standard curve of RLIA in the range of 0-15% of asialotransferrin, is similar to that of the curve obtained from serum samples in the range of 2-16% of serum desia-

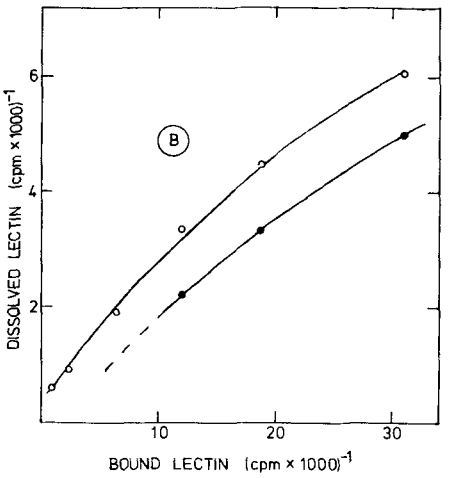

Fig. 4 B. Solubilization of bound lectin from the antitransferrin-transferrin complex recorded in Fig. 4 A. The penultimate (open circles) and final (filled circles) washing were analyzed for lectin that had been solubilized from the pelleted gel (one determination).

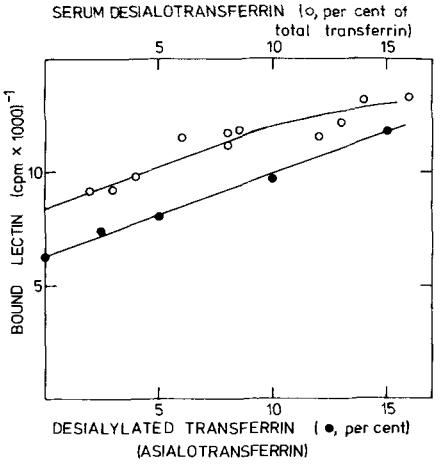

Fig. 5. Radio-lectin immunoassay of terminal galactose in transferrin from standard solutions containing 50 µg of transferrin with the indicated proportion of the completely desialylated form (filled circles) or from 100 µl of serum (open circles) containing transferrin with the indicated proportion of the desialotransferrin component of pI 5.7 (cf. Fig. 2 A-B). The transferrin was attached to 10 µl of antitransferrin Sepharose followed by equilibration with 80 µg of ^{125}I-Crotalaria lectin. The excess lectin was removed by three washings of 0.5, 1.0 and 2.0 ml respectively (mean of two determinations).

lotransferrin of pI 5.7 (2 expts.). This "physiological" serum transferrin component is the one that is of clinical interest in connection with alcoholism. In the experiment illustrated in Fig. 5, 6-12 µg out of 80 µg of lectin bound to the gel. A resolving power of 20 pmoles of asialotransferrin could be deduced from this experiment.

The sialic acid-binding lectin from *Limulus polyphemus* was also successfully

used for determining the nature of the terminal sugar residues of transferrin (Fig. 6 A and B). As expected, transferrin bound significantly more of this lectin than did asialotransferrin. However, the asialotransferrin-antitransferrin complex also bound significant amounts of this lectin. This is to be expected, since antibodies contain sialic acid residues and thereby contribute to the binding of Limulus lectin. The levels of bound lectin using transferrin from sera containing 3, 8, 16 and 21% of the pathological component did not differ.

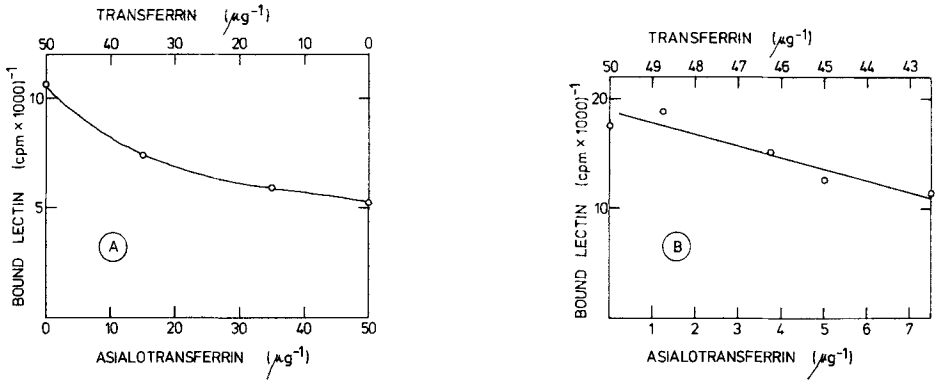

Fig. 6 A and B. Radio-lectin immunoassay of terminal sialic acid in transferrin using sialic acid binding ^{125}I-labelled lectin from *Limulus polyphemus*. After equilibration of 10 µl of antitransferrin-Sepharose gel with 50 µg of transferrin composed of the indicated amounts of native transferrin and fully desialylated asialotransferrin, the gel was washed and incubated with 25 µg of Limulus lectin with added tracer. The gels represented in Fig. 6 A and B were not washed identically.

DISCUSSION

The present results show that radio-iodinated lectins can be used for determining the proportions of different terminal sugars in transferrin bound to a solid phase via antitransferrin. This method is suitable for the analysis of terminal carbohydrates in immunologically defined glycoproteins displaying microheterogeneity in the glycosidic group(s) when the latter is not *per se* an antigenic determinant. The method opens new possibilities for investigating microheterogeneity arising from different neutral sugars, which at present cannot be differentiated by other means than large scale preparation followed by conventional analysis. The simplicity of the method will greatly facilitate the study of the microheterogeneity of glycoprotein material from body fluids particularly where such microheterogeneity has been correlated with or is under suspicion in connection with clinical disorders.

Alcoholism is a well-documented example of a clinical condition which is associated with the presence of modified sugar residues on a serum protein.

Patients with a history of prolonged and excessive alcohol consumption have 9.5 ±3.7% (S.D.) of a desialotransferrin component of pI 5.7, while normal subjects have 3.7 ±1.5% (S.D.) of this component, relative to total serum transferrin. The band having an isoelectric point of 5.7 is normally a minor serum transferrin component. It appears between two more acidic and two more basic bands when analyzed by isoelectric focusing of partially neuraminidase-digested transferrin, thus indicating that the desialotransferrin component found in serum has two sialic acid residues while fully sialylated transferrin has at least four residues (10,14,15). It is therefore interesting that a standard RLIA curve of galactose from a limited number of serum samples with "native" desialotransferrin concentrations ranging from 2 to 16% of total transferrin has a similar slope to that of a standard curve obtained from artificially desialylated asialotransferrin of concentrations ranging from 0 to 15% of added transferrin.

This finding is expected if each serum desialotransferrin molecule of pI 5.7 has lost one sialic acid molecule per glycosidic group. (There are at least two glycosidic groups in transferrin (14)). If this were the case, one glycosidic residue would be capable of binding one Crotalaria lectin molecule irrespective of the relative orientation of the transferrin and antitransferrin molecules. Furthermore, the two galactose residues in each of the two glycosidic groups per transferrin molecule are separated by only 5 sugar residues while the molecular weight of the Crotalaria lectin is 120 000 D (4). Consequently, for steric reasons, only one lectin molecule per glycosidic group can be expected to bind, even if both of the two galactose residues, normally shielded by sialic acid, are exposed. This interpretation is supported by the finding that sialic acid-binding lectin did not differentiate between the different serum concentrations of desialotransferrin of pI 5.7, although the assay conditions with this lectin were shown to resolve asialotransferrin concentrations in the clinically interesting range.

The present results support assumptions that the "physiological" serum desialotransferrin of pI 5.7 has lost two sialic acid residues. Furthermore, it is apparent that in the desialotransferrin, the terminal sialic acid is lost exposing galactose thus providing a basis for its quantification with galactose-binding lectin.

Transferrin is interesting in that it represents a known example of a correlation between glycosylation pattern of an immunologically defined serum polypeptide and a clinical condition. If more such examples are found in the future, the present method, as well as its modifications, will presumably become of general interest. The method may be modified whereby the labelled lectin that remains unbound after incubation with the antibody-antigen gel can be assayed, or the labelled bound lectin is resolubilized from the antibody-antigen gel as a final step. Furthermore, it would be possible to assay lectins that are label-

led to emit electromagnetic radiation under defined conditions, such as, for example, fluorescent markers or other radioactive markers.

The basic difficulties in using labelled lectins for assessing glycoprotein sugars according to the present method originate in the equilibrium binding of the components. This necessitates that the assay conditions are standardized regarding buffer composition, volumes, temperature and time of incubation and of washing. It is advisable that the affinity gel is saturated with the component of interest and that each assay contains serum components of defined sugar microheterogeneity covering the clinically interesting range. In such a case, a standard curve with defined slope and background levels of adsorbed lectin can be constructed.

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