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Determination of Sialic Acid Residues in Transferrin by Oxidative-Reductive Immunoassay

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ABSTRACT

A method is described for the determination of sialic acid residues in glycoproteins displaying microheterogeneity in the sugar residue. The new method is based on combining an oxidative-reductive step with binding of the glycoprotein to an immunoadsorbent. After a mild oxidation with sodium meta-periodate the sugar is reduced with labeled sodiumborotritide. The chemical modification of the sugar residues does not seem to impair the binding of glycoprotein to the immunoadsorbent. The procedure, which has been elaborated for human transferrin, can be carried out in the presence of other substances in body fluids.

INTRODUCTION

Combined physiocochemical separation techniques like isoelectric focussing and chromatofocussing are precise but time-consuming methods that are not suitable for use on a large multi-sample scale basis. Such methods also require expensive equipment and chemicals as well as skilled manual guidance. When complex mixtures are analyzed, the two methods generally have to be complemented with some kind of identification of the investigated protein. For these reasons such methods are not always suitable for routine analytical use on a microscale basis.

Therefore, new methods are needed that are compatible with rapid and simultaneous processing of a large number of samples, and which permit quantitative measurements.

For example, the microheterogeneity of glycoproteins mostly involves sialic acid and is a statistical phenomenon, where the average content of sialic acid usually gives more information than the relative amount of one particular isoelectric component out of several. In such cases no useful information is lost by recording average concentrations of sialic acid.

Recently, a method has been presented having the desired properties, the rational name of which is Radio-Lectin Immunoassay (1). However, a major drawback of methods involving lectins is that most lectins bind with low

affinity to their corresponding sugars. This means that the conditions have to be standardized with respect to several parameters that are difficult to control, such as the turbulence occurring when replacing the buffer during washing the immunoadsorbent. Hence, there is an actual want of rapid and reliable methods for determination of sugar residues in glycoproteins. The present paper describes a simple and rapid method for determination of sialic acid residues in glycoproteins displaying microheterogeneity. The method is at least semiquantitative and will therefore replace in some instances qualitative procedures like isoelectric focussing.

MATERIALS

Buffer salts, citric acid and unlabeled sodium borohydride were reagent grade from Merck AG, Darmstadt, W. Germany. Tris, transferrin, neuraminidase, albumin and N´N´-dimethylformamide were from Sigma, St Louis, Miss. USA. Affi-Gel 10 was from Biorad Laboratories, Richmond, Calif. USA, antitransferrin (nephelometric titer, 0.35 mg of antigen/ml) from Kallestad, Behringwerke, Frankfurt am Main, W. Germany, tritiated sodium borohydride (350 mCi/mmol) and Aquasol from NEN Chemicals, Dreieich, W. Germany. Blue Dextran and Sephadex G200 were from Pharmacia AB, Uppsala, Sweden.

METHODS

Preparation of Immunoadsorbent. Two ml of Affi-Gel 10 was washed 5 times with an ice-cold solution consisting of 20 mM Na_2HPO_4, 100 mM NaCl, pH adjusted to 7.5 with HCl (PBS, pH 7.5). Twenty mg of transferrin were dissolved in 1 ml of the same buffer, the gel suspended in a total volume of 3 ml and transferred to the transferrin solution. After incubation for 30 min at room temperature with gentle magnetic stirring, $CaCl_2$ was added and the coupling reaction continued for another 30 min at room temperature. 0.2 ml of 1 M ethanolamine - HCl, pH 8.0 were then added followed by incubation with stirring for 1 h at room temperature, the gel transferred to a Pasteur pipette and washed overnight with PBS, pH 7.5 (200 ml) and then with 100 ml of 150 mM NaCl phosphate citrate buffer prepared by adding solid Na_2HPO_4 to 10 mM citrate until the pH reached 2.8 (phosphate-citrate, pH 2.8). Subsequently it was washed with 100 ml of PBS, pH 7.5, containing 1 M NaCl, and then again with phosphate-citrate. Finally it was equilibrated with PBS, pH 7.5, and 20 ml of antitransferrin in the same buffer were recirculated for 3 h or overnight at $4^{o}C$, the column washed with PBS, pH 7.5 containing 1 M NaCl followed by elution of the antitransferrin by the phosphate-citrate buffer. Approximately 10 mg of antitransferrin were obtained, judging from A_{280} and with transferrin as a standard. The eluted antitransferrin was neutralized and

concentrated to approximately 10 mg/ml in PBS, pH 7.5. One ml of Affi-Gel in 2 ml of PBS, pH 7.5 was then added and the coupling reaction allowed to take place with gentle stirring at room temperature for 2h. Thereafter, 0.2 ml of 1M ethanolamine -HCl, pH 8.0 were added and the incubation continued for 1 h at room temperature. Finally, the gel was transferred to PBS, pH 7.5 as a 50% packed gel suspension.

Neuraminidase Digestion of Transferrin. Twenty mg of human transferrin containing approximately 0.9 μmoles of sialic acid were dissolved in 1 ml of 0.1 M sodium phosphate buffer, pH 6.5 and incubated with 0.5 U of neuraminidase from Dactylium Dendroides for 30 min at 37°C. (One unit of neuraminidase is defined as the amount necessary to liberate 1.0 μmole of sialic acid per min at pH 5.0 and at 37°C).

After digestion, Blue Dextran was added as a marker for the void volume and the sample fractionated in PBS, pH 7.0 (20 mM Na_2HPO_4, 150 mM NaCl, pH adjusted with HCl) on a Sephadex G-200 column with a diameter of 1.2 cm and a length of 38 cm. The transferrin was eluted and located by its absorption at 280 nm. The concentration of transferrin was adjusted to 1 mg/ml with native transferrin as a standard. Mixtures of the neuraminidase-digested transferrin and transferrin were then used for comparative analysis of sialic acid using the described method.

Acid Hydrolysis of Transferrin. Ten mg of transferrin were hydrolyzed in 1 ml of 1N H_2SO_4 at 80°C for 1h, the solution neutralized with K_2HPO_4 and the asialotransferrin separated on the Sephadex G200 column described above.

Comparative analysis of sialic acid. The analysis of sialic acid in transferrin was carried out by combining an oxidative-reductive step with binding the glycoprotein to antitransferrin immunoadsorbent. The volume and concentrations of the various solutions varied according to the sequence in which these steps were performed and the presence or absence of other glycoprotein components than transferrin. The following solutions were used: PBS, pH 7.0: 20 mM Na_2HPO_4, 150 mM NaCl, pH adjusted with HCl.

Tris-glycine-glycerol: 0.1 M Tris-base adjusted to pH 8.0 with 0.1 M glycine and glycerol added to 0.1 M.

Borotritide solution: Tritiated sodium borohydride dissolved in 0.1 M NaOH/N´N-dimethylformamide (1:1) to a final concentration of 1.78 mCi/ml.

Gel protecting reagent. This mixture protected the solid phase gel, by inhibiting the reaction of labeled compounds with the particular type of gel used, and was prepared as follows: One volume of PBS, pH 7.0 containing 2 mM sodium meta-periodate was incubated with one volume of Tris-glycine-glycerol for 30 min at room temperature, and then with one volume of 50% 0.1 M NaOH and 50% N´N´-dimethylformamide containing 2mM sodium borohydride.

Washing solution PBS, pH 7.5, containing 10% N´ N´ -dimethyl-formamide. 0.1 mg of transferrin were routinely used in the assay. The oxidative step was always performed after the solutions had reached $0^{o}C$ in an ice-bath. Sodium meta-periodate (2mM) in PBS, pH 7.0 was added for oxidation which was allowed to proceed for 10 min. The reactions were quenched with Tris-glycine-glycerol and then either reduced with borotritide or washed and equilibrated with 20 µl of immunoadsorbent suspension. The details are described in the Figure legends. After reduction, the gel was washed free of excess reagent, transferred to a scintillation vial and counted by liquid scintillation using 10 ml of Aquasol.

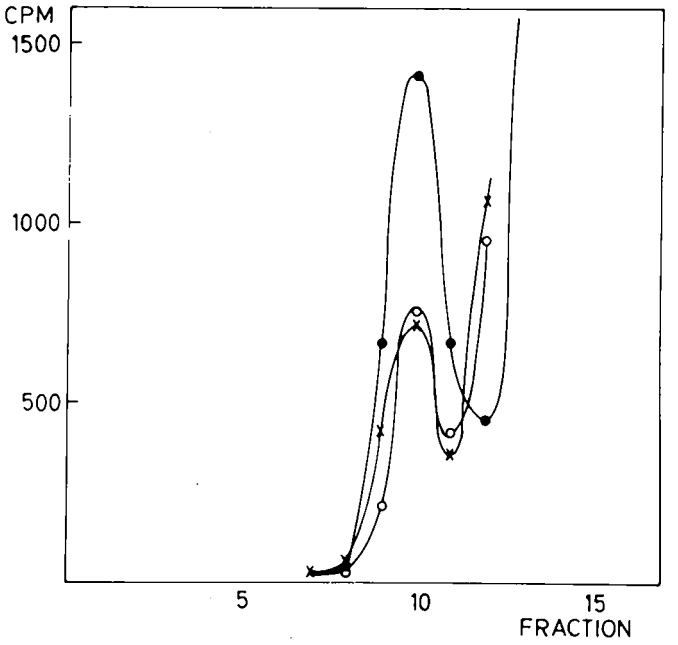

Fig.1 0.1 mg of transferrin was dissolved in 0.1 ml of PBS, pH 7.0 and 0.1 ml of the same buffer containing 2 mM sodium metaperiodate was mixed at ice-temperature and incubated for 10 min. A mixture from 0.1 M glycine and 0.1 M Tris, pH 8.0 containing 0.1 M glycerol was then added followed by incubation with stirring at room temperature for 30 min. The mixture was kept in ice for 30 min and 0.1 ml sodium borotritide, 1.78 mCi/ml, in 50% 0.1M NaOH/50% N´N´-dimethylformamide added. After incubation on ice for 30 min the labeled transferrin was separated from excess reagent by passing the mixture over a Sephadex G-25 column. The total fractions were mixed with Aquasol and counted for tritium, (•) transferrin, (o) neuraminidase-digested transferrin, (x) acid-hydrolyzed transferrin (0.1 N H_2SO_4 for 1h at $80^{o}C$).

RESULTS AND DISCUSSION

One hundred μg of transferrin or desialylated transferrin were labeled with sodium meta-periodate-borotritide and then separated from excess labeled borotritide by fractionation on Sephadex G-25 columns (PD-10, Pharmacia AB) (Fig. 1). Subsequent estimation of the surface area under the void peak containing the labeled protein indicated that sialotransferrin incorporated approximately 3 times more label than asialotransferrin. The asialotransferrin which was prepared by neuraminidase digestion or hydrolysis in 0.1 N H_2SO_4 at 80°C for 1 h gave similar results. The oxidation with periodate is not exclusively specific for sialic acid (2) and therefore, a certain background activity in the absence of sialic acid must be anticipated.

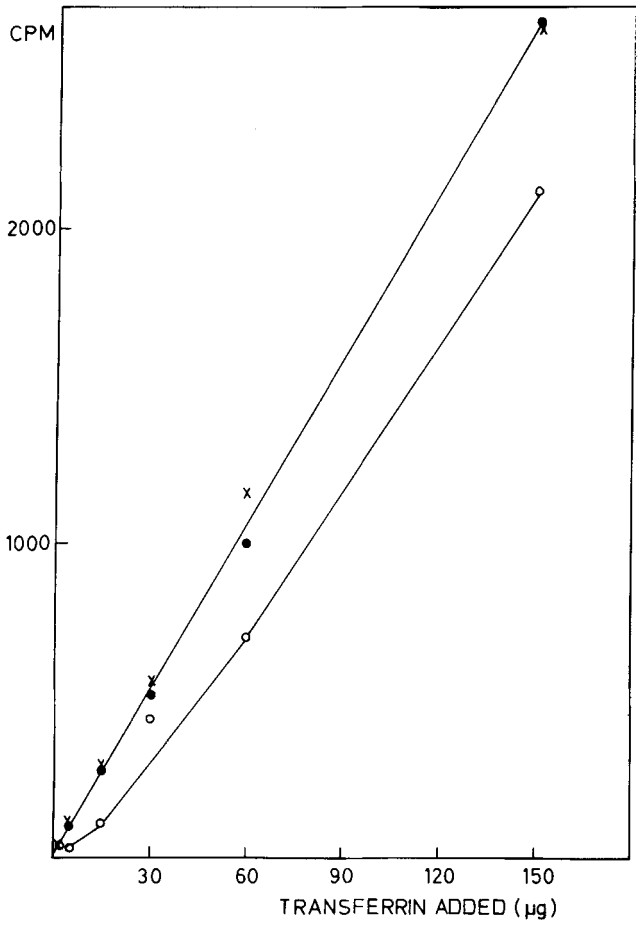

Fig. 2A Estimation of transferrin-binding capacity of the antitransferrin gel. Transferrin was labeled by oxidation with 1 mM $NaIO_4$ at pH 7.0, 0°C for 10 min followed by quenching with 0.1 M glycine-Tris containing 0.1 M glycerol and tritiation with sodium borotritide. The labeled sialotransferrin was then transferred to PBS pH 7.5 by gel filtration on Sephadex G-25 (PD-10 column) and diluted to 1 mg/ml and a specific activity of $5x10^5$ cpm per mg. Samples of 2-150 μg of labeled transferrin were then diluted to equal volume and incubated with 20 μl of 50% (V/V) antitransferrin gel suspension, or control gel suspension, the mixture diluted to 3 ml with PBS pH 7.5 and the gel pelleted after which 0.1 ml of the supernatant was transferred to a scintillation vial and counted with Aquasol using the tritium channel in a liquid scintillation counter. The points represent the radioactivity contained in the supernatant, in (x) test tube only; (●): Affi-Gel -10, treated as for binding antitransferrin; (o): Affi-Gel coupled to antitransferrin.

Labeled sialotransferrin was prepared and used to assess the antigen-binding capacity of the antitransferrin gel in relation to unspecific adsorption (Fig 2A,B). It is evident from Fig 2A, where a sample from the supernatant not reacting with the gel is analyzed, that virtually no background binding of labeled transferrin occurred, neither to the gel nor to the test tube. Ten µl of the antitransferrin gel bound 10-25 µg of transferrin when equilibrated with a total amount of 15-150 µg in solution, judging from the control standard curve with no transferrin antibodies (Fig 2A). When 100 µg of transferrin were added, approximately 22 µg bound to the gel.

In Fig 2B where the amount of gel was varied, figures from 18-36 µg of bound transferrin per 10 µl of antitransferrin gel (20 µl of gel suspension) were obtained. The higher values probably represented a saturation phenomenon in the equilibrium binding of the antigen-antibody during the short (30 min) incubation time used. The results clearly show that approximately 20 µg of transferrin bound to 10 µl of gel.

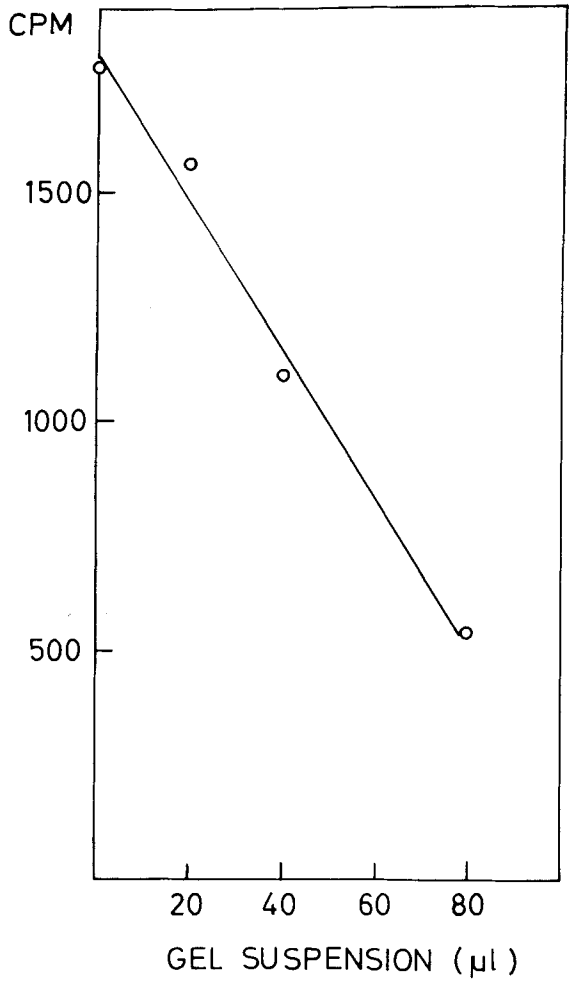

Fig. 2B Same experiment as in (A) except that the amount of gel suspension was varied and the amount of labeled transferrin kept constant, 10.0 µg. The radioactivity remaining in 0.1 ml of supernatant after incubation of the mixture, dilution to 3 ml, and pelleting was measured.

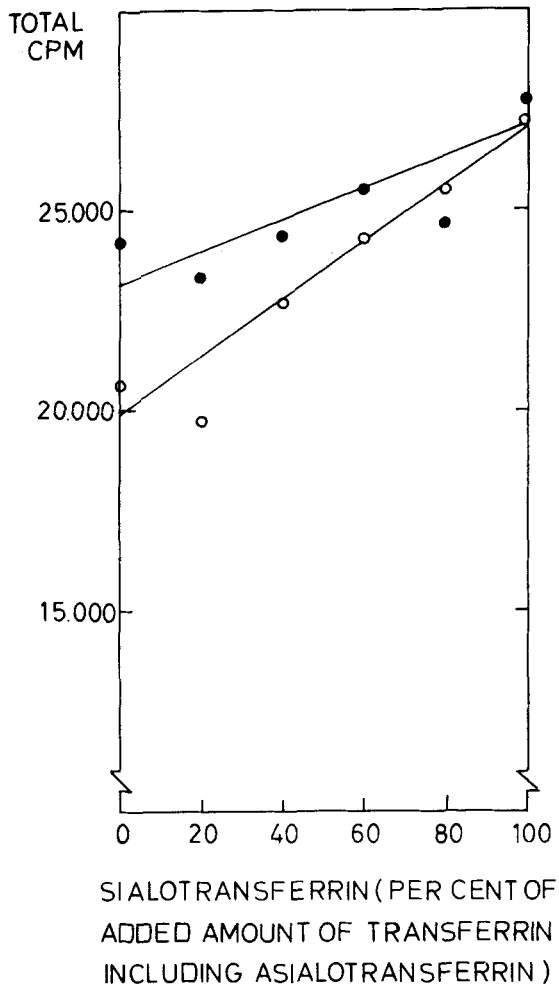

Fig. 3A Combination of oxidation-reduction with binding of transferrin followed by separation of the labeled immunocomplex in a single step.
The procedure in Fig 2 was followed, PBS, pH 7.5 was added to a total volume of 0.5 ml and 20 µl of the antitransferrin gel added and the mixture incubated with vigorous shaking for 30 min at room temperature. After incubation, the gel was allowed to sediment, the supernatant aspirated and the gel washed 5 times with 5-10 ml of washing solution. The final supernatant was checked to assure close to background-levels of radioactivity: o-o Transferrin only, ●-● Transferrin with 25 µl of serum.

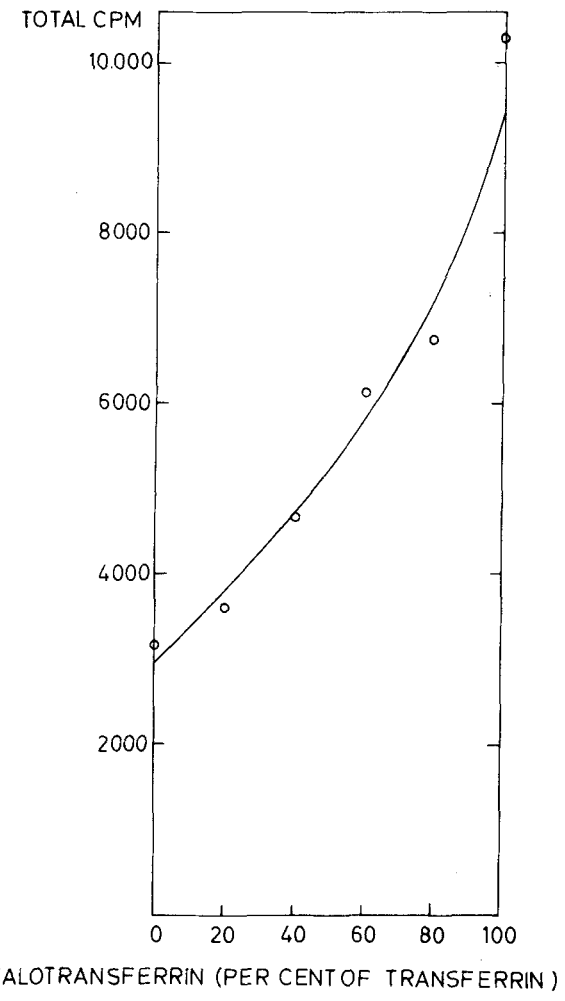

Fig. 3B Combination of oxidation-reduction with binding of transferrin followed by separation of the labeled immunocomplex in a single step.
The concentration of Trisglycine-glycerol was reduced to half by diluting with water, otherwise the oxidation-quenching-reduction sequence followed. The antitransferrin gel was first incubated with gel-protecting reagent at 0°C. The composition of this reagent is described in the Methods section. Thereafter the transferrin was transferred to the gel suspension and the mixture incubated for 60 min at room temperature followed by washing.

In Fig 3A-C, the oxidation-reduction of transferrin was combined with binding the glycoprotein to the immunoadsorbent and separated from the mixture in a single step. In 3A, the procedure in Fig 2 was followed and the glycoprotein incubated with the gel in the presence of excess radioactive products. The final supernatant after washing was checked to assure close to background levels of radioactivity. The high background activity with asialotransferrin only, was due to chemical reaction of the radioactive products with the gel matrix, since it was observed with a similarly treated gel lacking antitransferrin. The high background activity was not reduced by treating the gel with 1 mM sodium borohydride prior to exposure to the radioactive products. The two curves in Fig 3A represent transferrin of various sialic acid content with or without 25 µl of serum added before the oxidative step. As expected the addition of serum containing sialylated transferrin increased the labeling of the samples with a low content of transferrin and a high content of asialotransferrin. The concentration of transferrin in the serum sample can be estimated to be 3.4 g/l from the ratio of diluted/undiluted sample since the total amount of transferrin added exceeded the transferrin-binding capacity of the immunoadsorbent. This was within the normal range (4), demonstrating that the method was reliable quantitatively. In Fig 3B, the gel was first incubated with the gel-protecting reagent before incubation with the transferrin mixture containing radioactive products. This decreased the background radioactivity significantly. The observed background activity in Fig 3B, using asialotransferrin only, was approximately 1/3 of the incorporation of tritium in the fully sialylated transferrin. This percentage was comparable to the unspecific incorporation into asialotransferrin as demonstrated in Fig 1, which was most probably due to incorporation of label into other sugar residues than sialic acid.

In Fig 3C, the transferrin was first oxidized, then bound to the antitransferrin gel, washed, and finally incubated with borotritide and the activity incorporated into the solid phase transferrin measured. The experiments illustrated in Fig 3 show that the method presented can be used to assess sialic acid content in a glycoprotein and that the sequence of the various incubations is not critical to achieve a comparative analysis of the sugar. The critical step is the separation from a mixture of the immunocomplex containing the labeled sugar.

The method presented may be used as an alternative to isoelectric focussing when a large number of samples are processed simultaneously to obtain information on the average content of sialic acid in a glycoprotein.The method does not presently seem to be sensitive enough to measure the previously reported microheterogeneity of serum transferrin in connection with

alcohol abuse which at best involves a difference of about 10% of the amount of transferrin and 5% of the total amount of transferrin-bound sialic acid (3).

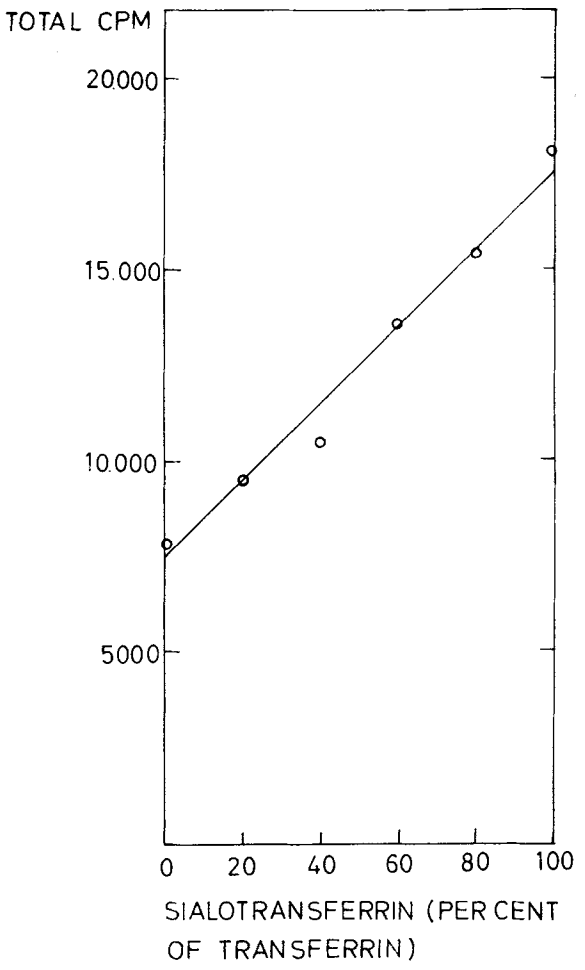

Fig. 3 C Combination of oxidation-reduction with binding of transferrin followed by separation of the labeled immunocomplex in a single step. The transferrin was first oxidized, then incubated with Trisglycine-glycerol for 60 min at room temperature and then with 20 µl of gel suspension for another 60 min. Thereafter the gel was washed, first with PBS containing 1% albumin and then with PBS pH 7.0 0.1 ml of borotritide solution was then added and the volume made 0.6 ml with PBS, pH 7.0 followed by incubation for 5 min at room temperature, washing and measuring bound radioactivity.

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