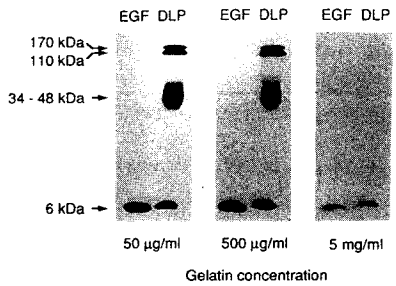

FIG. 1. Effect of the gelatin concentration in the membrane coating solution on Western blot analysis of EGF. Rat submaxillary gland EGF (10 ng) and a tissue extract prepared from androgen-primed rat dorsolateral prostate (DLP, 60 μg protein) were subjected to SDS–polyacrylamide gel electrophoresis under reducing conditions. After electrophoresis, the proteins were transferred to PVDF membranes treated with the indicated concentrations of gelatin solution for 10 min. The membranes were fixed with a 3.7% (w/v) formaldehyde solution after blotting. EGF bound to the membranes was visualized as described in the text.

Tween 20 is frequently used in blotting analysis of proteins and nucleic acids to decrease background staining due to nonspecific binding of antibodies to various membranes. Inclusion of 0.1% (w/v) Tween 20 in the washing buffer (Buffer 1) significantly decreased the chemiluminescence signals of both LMW-EGF and HMW-EGFs (data not shown). This result indicates that the binding of proteins to PVDF membranes is susceptible to the detergent even after fixation with formaldehyde.

In conclusion, we suggest the following conditions for the analysis of LMW-EGF: (a) treat PVDF membranes with a 500 μg/ml gelatin solution, (b) carry out electroblotting for 10 min (the duration depends on the

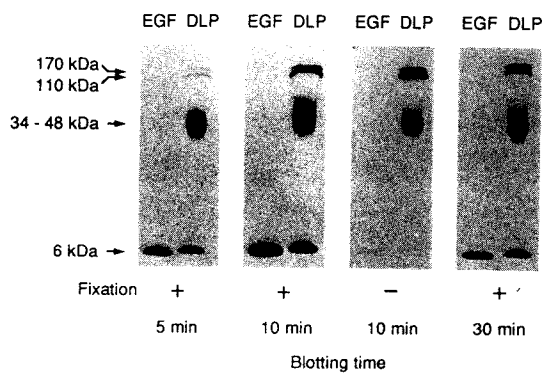

FIG. 2. Effect of the blotting time on Western blot analysis of EGF. Rat submaxillary gland EGF (10 ng) and a tissue extract prepared from androgen-primed rat dorsolateral prostate (DLP, 60 μg protein) were subjected to SDS–polyacrylamide gel electrophoresis under reducing conditions. After electrophoresis, the proteins were transferred to PVDF membranes treated with a 500 μg/ml gelatin solution for 5, 10, or 30 min. All the membranes except for one were fixed with a 3.7% (w/v) formaldehyde solution after blotting. EGF bound to the membranes was visualized as described in the text.

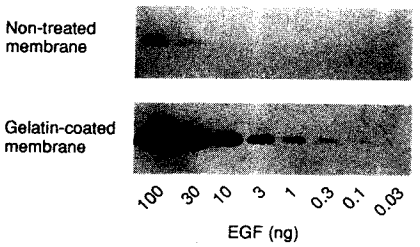

FIG. 3. Effectiveness of the use of gelatin-coated membranes in Western blot analysis of EGF. Rat submaxillary gland EGF (100–0.03 ng) was subjected to Western blot analysis as described in the legend to Fig. 1 using a nontreated membrane and a membrane treated with a 500 μg/ml gelatin solution.

blotting conditions used), (c) fix the membranes with formaldehyde after blotting, and (d) do not include Tween 20 in the washing buffer. The optimized conditions made it possible to detect 0.1–0.3 ng of rat LMW-EGF (Fig. 3). Although the conditions are not optimum for the detection of HMW-EGFs, as described above, it is possible to analyze LMW- and HMW-EGF species simultaneously on a single membrane with this method.

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Modified Assay for the Measurement of Biotin in the Presence of Heparin

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Avidin–biotin interaction has been applied to a wide variety of analytical problems including the analysis of

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proteins (1). Glycoproteins can also be labeled with a hydrazide derivative of biotin following the peroxidation of their moieties (2). Oligosaccharides have recently been biotinylated at their reducing end using 2-amino-(6-biotinyl) pyridine (3). Glycosaminoglycan heparin has been biotinylated after activation of its hydroxyl groups with cyanogen bromide, reaction with diaminohexane, and coupling to an *N*-hydroxysuccinimidyl ester of biotin (4). The biotin incorporated into macromolecules can be quantified using an avidin–HABA[2] (hydroxyazobenzoic acid) assay. This assay has been used for the determination of the degree of biotinylation of proteins and is based on the principle that HABA binds to avidin at the same site as biotin and can be displaced by biotin. When HABA binds to avidin, it loses planarity and forms its hydrazone tautomer (5). When released by biotin, it regains planarity and its absorbance in solution changes. The displacement of HABA by biotin causes a change in the absorbance that is proportional to the concentration of added biotin (5). The interaction of HABA with avidin is weaker than the interaction of biotin with avidin which makes the displacement of HABA by biotin essentially an irreversible process.

Heparin is a strongly anionic glycosaminoglycan that binds to a number of important proteins including coagulation proteins, chemokines, and growth factors (6). Initial studies in our laboratory showed that heparin severely interfered with the HABA assay hindering the accurate determination of biotin in samples containing heparin and heparin–biotin conjugates. In this note we describe a method to eliminate heparin interference in the avidin–HABA dye displacement assay for biotin.

Experimental procedures. Heparin (166 USP units/mg) was obtained as the sodium salt from Celsus Laboratories, Inc. (Cincinnati, OH). D-(+)Biotin was from Pierce Chemical Co. (Rockford, IL). The Fluoreporter biotin labeling and detection kit with avidin–HABA (avidin–hydroxyazobenzoic acid complex) reagent was from Molecular Probes Inc. (Eugene, OR). All other reagents were from either Fisher Scientific (Pittsburgh, PA) or Aldrich Chemical (St. Louis, MO). Spectrometric measurements were made on a Shimadzu Model UV-160 spectrophotometer.

Biotin was first measured in the absence of heparin using the avidin–HABA assay. The assay was carried out by aliquoting 500 μl of the avidin–HABA reagent into glass test tubes. Biotin (0 to 2 μg) was added to the test tubes as a solution in 10 μl of water. The solution was mixed using a vortex mixer and the absorbance was measured at 500 nm against a reagent blank.

Biotin was next measured in the presence of increasing amounts of heparin using the avidin–HABA assay. Biotin (2 μg) was added to each test tube containing 500 μl of the avidin–HABA reagent as a solution in 10 μl of water. The solution was mixed using a vortex mixer and the absorbance was measured at 500 nm against a reagent blank. Heparin (0 to 20 μg in 10 μl of water) was added to each test tube and the absorbance was measured as above. In the modified assay, 100 μl of 1 M sodium bicarbonate solution was added to each tube in addition to the above solutions and the assay was carried out in a similar fashion.

Results and discussion. In the absence of heparin, the avidin–HABA assay gave an absorbance that decreased linearly with an increasing concentration of

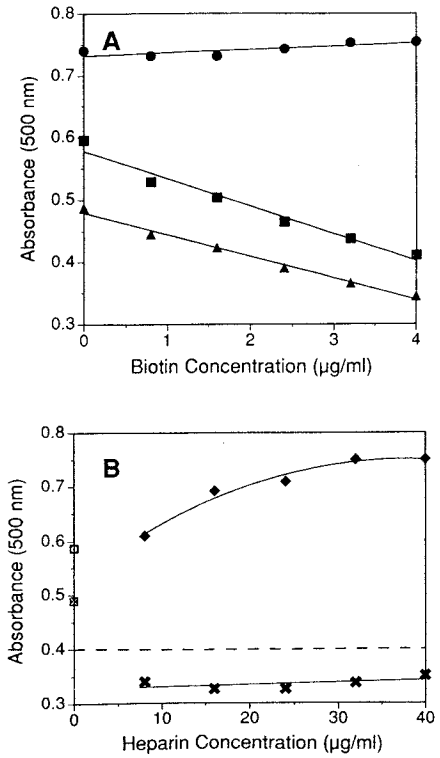

FIG. 1. (A) Absorbance is plotted as a function of biotin concentration. A standard assay (■) gives a linear curve between 0 and 4 μg/ml biotin with a slope of -0.041 $\Delta A_{500}/\mu g$ ml^{-1} biotin. The addition of heparin (40 μg/ml) gives an elevated absorbance value and a slope of $+0.005$ (●). The addition of sodium bicarbonate to a concentration of 0.16 M affords a linear curve (▲) with a slope of -0.036 $\Delta A_{500}/\mu g$ ml^{-1} biotin. (B) Absorbance as a function of heparin concentration. The standard biotin assay was performed in the presence of 4 μg/ml biotin with heparin concentration of 0 to 40 μg/ml (♦) and gave a nonlinear curve. A standard biotin assay with a concentration of 4 μg/ml biotin and no added heparin gives the absorbance of 0.4 as indicated by the dotted line. In the absence of biotin and heparin, an absorbance of 0.59 was observed (□). A modified biotin assay containing 4 μg/ml biotin and 0.16 M sodium bicarbonate gave a linear curve (✖) with a slope of 0.00 $\Delta A_{500}/\mu g$ ml^{-1} heparin from 8 to 40 μg/ml of added heparin. In the absence of biotin and heparin but in the presence of 0.16 M sodium bicarbonate, an absorbance of 0.49 (⊠) was observed.

[2] Abbreviation used: HABA, hydroxyazobenzoic acid.

biotin (Fig. 1A). When heparin was added, the absorbance was nearly constant with increasing biotin concentration and identical to that of the blank (0 μg/ml biotin). The level of interference by heparin was determined by constructing a standard curve with a solution containing increasing amounts of heparin (7 to 40 μg/ml) but containing a constant amount (4 μg/ml) of biotin (Fig. 1B). An increase in absorbance was observed on addition of increasing concentrations of heparin. Even small amounts of heparin (7 μg/ml corresponding to ~1 USP unit of heparin) produced significant changes in absorbance. When the same standard curve (biotin at 4 μg/ml, heparin at 7 to 40 μg/ml) was constructed after the addition of sodium bicarbonate to a concentration of 0.16 M, the interference due to heparin disappeared. Although no increase in absorbance was observed with increasing heparin concentrations, the samples containing sodium bicarbonate gave an absorbance (A_{500} = 0.49) below that of the blank containing the same concentrations of biotin but no sodium bicarbonate (A_{500} = 0.59). A new standard curve measuring increasing amounts of biotin was then constructed in presence of both heparin (40 μg/ml) and sodium bicarbonate (0.16 M) (Fig. 1A). While the absorbance at the initial point (biotin 0 μg/ml) was again depressed (A_{500} = 0.49), absorbance decreased linearly with increasing concentrations of biotin. The slope obtained in the absence of heparin and sodium bicarbonate ($-0.041\ \Delta A_{500}/\mu g\ ml^{-1}$ biotin) was nearly the same as the slope obtained ($-0.036\ \Delta A_{500}/\mu g\ ml^{-1}$ biotin) in the presence of heparin (40 μg/ml) and sodium bicarbonate (0.16 M).

The ability of sodium bicarbonate to remove the interference by heparin in this assay may be attributable to the strongly anionic nature of heparin (6). One of the components in the avidin–HABA solution interacts with heparin causing the solution to become turbid resulting in an increase in absorbance at 500 nm. Sodium bicarbonate eliminates heparin interference by eliminating this turbidity. The modified assay described represents a convenient method for determining the degree of biotinylation of a protein or a carbohydrate in the presence of heparin and might possibly reduce the interference caused by other glycosaminoglycans or acidic polysaccharides.

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Determination of the Linkage Positions of Reducing-End Residues of Oligosaccharides by Partial Periodate Oxidation of Pyridylaminated Derivatives

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Pyridylamino (PA)[2] sugars are amenable to sensitive analysis (1), and we proposed a method (2) for determining the linkage positions of reducing-end residues of PA-oligosaccharides by using Smith degradation (3,4). Linkage-position analysis of PA-disaccharides was accomplished by HPLC of the fluorogenic Smith degradation products, produced by complete periodate oxidation for 40 h at 4°C followed by reduction and hydrolysis. In the cases of PA–hexose residues, additional partial periodate oxidation for 15 min at 0°C and product analysis was necessary for differentiation of 4- and 6-substitution because complete oxidation gave the same fluorogenic compound, $CH_2OH–CH_2–$PA (2).

The flexible PA-reducing-end residues are oxidized by periodate more rapidly than the rigid pyranose-ring residues. It is, therefore, expected that the linkage positions of the PA-reducing-end residues can be determined by analyzing the fluorogenic products of partial periodate oxidation only, even though the pyranose-ring residues are intact. This paper describes a simple linkage-position analysis using partial periodate oxidation.

Materials and Methods

Materials. PA-disaccharides, PA-L-threose, PA-L-erythrose, PA-DL-glyceraldehyde, PA-glycolaldehyde, PA-2-acetamido-2-deoxy-D-xylose, PA-2-acetamido-2-deoxy-L-threose, and PA-2-acetamido-2-deoxy-D-glyc-

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[2] Abbreviations used: PA, pyridylamino; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Fuc, fucose; Glc, glucose; Man, mannose; BI6F, Galβ1–4GlcNAcβ1–2Manα1–6(Galβ1–4GlcNAcβ1–2Manα1–3)-Manβ1–4GlcNAcβ1–4(Fucα1–6)GlcNAc-PA. Unless otherwise noted, all sugars are of D-configuration except fucose.

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