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THE FUNCTION AND COMPOSITION OF THE CARBOHYDRATE PORTION OF HUMAN URINARY ERYTHROPOIETIN.

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THE FUNCTION AND COMPOSITION OF THE CARBOHYDRATE PORTION OF HUMAN URINARY ERYTHROPOIETIN

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE DIVISION OF BIOLOGICAL SCIENCES
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BY

MARGARET ANN SMITH DORDAL

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

ABBREVIATIONS

ACTH	Adrenocorticotropin
Epo	Erythropoietin
hCG	Human Chorionic Gonadotropin
HPLC	High Pressure Liquid Chromatography
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LH	Luteotropin
NAN-lactose	N-Acetylneuramin-lactose
SDS	Sodium Dodecyl sulfate
TFA	Trifluoroacetate
THAM	Tris-hydroxymethylaminomethane
TMS	Trimethylsilyl
TSH	Thyrotropin

CHAPTER I

INTRODUCTION

Erythropoietin (epo) is the principal hormone regulating mammalian erythrocyte development (1). The epo purified from the urine of human patients with aplastic anemia is a glycoprotein with an apparent molecular weight of 39,000 (2,3). Until now, the limited quantities of purified protein available have permitted only preliminary studies of the carbohydrate moiety. The present study is an attempt to define the importance of the carbohydrate to the biological and immunological activities of epo. Additional data about the composition of the carbohydrate moiety are also presented.

Epo is absolutely required for the normal initiation and maintenance of erythropoiesis in mammals (1). The primary site of epo synthesis is the kidney (4,5). In humans a major complication of chronic renal disease or bilateral nephrectomy is anemia. Injection of exogenous epo can increase erythropoiesis in such patients (1). Injection of exogenous epo also induces increased erythropoiesis in rats and mice, as measured by either histological changes in the bone marrow (6) or increased incorporation of ^{59}Fe into erythrocytes in the whole animal (7). During erythropoiesis red cell precursors mature from a pluripotent stem cell to

the mature erythrocyte through a series of committed, unipotent stem cells, proerythroblasts, erythroblasts, and reticulocytes. Epo affects cells at several points in this path. When bone marrow cells are dispersed in semisolid medium in the presence of epo, stem cells will proliferate and differentiate, producing discrete colonies of erythroblasts. The colonies formed from early mouse stem cells require a week to mature and are clustered in groups called "bursts." The colonies formed from late stem cells mature in two days and are dispersed throughout the medium. Increasing doses of epo increase the size and number of colonies formed from both early- and late-stage committed stem cells (8,9). Increasing doses of epo also increase hemoglobin synthesis, an activity of early erythroblasts (8).

The earliest effect observed after adding epo to a bone marrow culture is an increase in RNA synthesis (10). Subsequently, total protein synthesis, iron uptake, and hemoglobin synthesis increase (11), as does DNA synthesis (12). Inhibition of DNA or protein synthesis does not prevent the stimulation of RNA synthesis by epo, but inhibition of RNA synthesis is sufficient to prevent the subsequent effects of epo (13,14).

Bioassays for epo are based on the ability of the hormone to stimulate iron uptake and heme synthesis in a dose-dependent fashion (7). In studies involving alteration of the carbohydrate portion of a glycoprotein hormone, *in vitro* assays are preferable to *in vivo* assays because the liver

contains a group of lectins which bind specific carbohydrate sequences and eliminate altered glycoproteins from the bloodstream very rapidly (15-17). Although desialylated epo retains full activity *in vitro*, it is inactive *in vivo*, presumably because it is cleared by the liver (3). Goldwasser, *et al.*, describe a suitable *in vitro* assay based upon the incorporation of ^{59}Fe into newly synthesized heme by rat bone marrow cultures (18). This bone marrow assay can detect additions of 0.001 unit of epo (1.4×10^{-21} g). One unit of epo is defined as the biological activity of one-tenth of the contents of an ampule of the International Reference Preparation, a lyophilized sample of crude human urinary epo.

Antibodies have been prepared against crude epo in rabbits and rats (19-21). Sherwood and Goldwasser developed a radioimmunoassay (RIA) which measures the displacement of pure [^{125}I]iodoepo from the crude polyclonal antibodies (20). For this assay, purified human urinary epo was iodinated with either Bolton-Hunter reagent, which alkylates amino groups (22), or Iodo-Gen, which modifies tyrosine residues (23). Although neither iodinated material had biological activity, both were bound to antibodies directed against epo and could be displaced by native epo. Asialoepo and native epo displaced similar amounts of [^{125}I]iodoepo. Most samples showed a good correlation between the activities measured by the RIA and by the bioassays. However, sera from two anemic patients with chronic

renal disease had much higher titers in the RIA than in the *in vivo* plethoric mouse assay. Gel permeation chromatography of these samples indicated that the reactive material was of much lower molecular weight than native epo.

Previous studies have shown that epo can be separated by hydroxylapatite chromatography into two forms, designated α- and β-epo. The two forms have identical apparent molecular weights, specific activities, and N-terminal amino acid sequences, as well as similar amino acid compositions, but exhibit different mobilities on polyacrylamide gel electrophoresis at pH 9 (2,24,25). α-Epo is eluted from hydroxylapatite at a lower ionic strength than β-epo and has a lower mobility at pH 9 (2). Heterogeneity in the carbohydrate portion of a glycoprotein can cause such differences in mobility on polyacrylamide gel electrophoresis (26).

The amino acids recovered in the compositional analysis account for only 40% of the mass of epo, suggesting that the hormone might be heavily glycosylated (25). The results of neuraminidase and galactose oxidase treatment imply that epo contains neuraminic acid as a terminal sugar and galactose which is exposed after neuraminidase treatment (3). One objective of the present study is to obtain a complete carbohydrate composition, both as a prerequisite to study of the structure of the carbohydrate and to determine whether there are differences in the composition of α- and β-epo which would account for the differences in physical behavior.

Sufficient supplies of purified epo to permit hormone replacement therapy would be clinically useful, especially in cases of anemia secondary to renal disease. Current supplies of purified epo are not adequate to permit extensive clinical trials of the hormone. Inducing synthesis of epo in bacteria or yeast by genetic cloning techniques presents an attractive alternative to purification from natural sources. However, bacteria are incapable of properly glycosylating proteins and yeast can produce only high-mannose oligosaccharides. For this reason, it is important to determine how much, if any, of the carbohydrate found in native epo is required for activity. Studies of other glycoproteins have revealed few consistent patterns in the effects of glycosylation on biological activity. Proteins such as soybean agglutinin (27), alkaline phosphatase (28), and human urinary colony-stimulating factor (29) remain fully active when deglycosylated, while others such as human chorionic gonadotropin (hCG) (30), rabbit IgG (31), and insulin receptor (32) lose part or all of their activity. Glycosylation may also alter either the stability (33,34) or the *in vivo* compartmentalization (15-17,35) of proteins. Goldwasser, *et al.*, have demonstrated that desialylation decreases the stability of epo to heat and trypsin digestion (3).

The relationship between the biological behavior of a glycoprotein and the structure of its carbohydrate may be examined by removing sugars from the intact protein, by

modifying sugars, or by synthesizing protein lacking sugar. The last approach is not presently feasible with epo because there is no system available in which the synthesis of epo can be altered and the resulting protein collected and studied. Except for galactose oxidase, most of the techniques which could be use to modify carbohydrate will also affect the polypeptide chain. In addition, chemical modifications generally will not distinguish between different sugars. Carbohydrate may be removed from glycoproteins either enzymatically or chemically. There are many glycosidases available representing a variety of substrate specificities (36). Hydrolysis in 0.1 M HCl at 80° for 1 hour will remove terminal neuraminic acids without hydrolyzing the polypeptide chain. Anhydrous hydrogen fluoride is reported to remove carbohydrate without cleaving peptide bonds or affecting the activity of ribonuclease or lysozyme (37).

When using deglycosylation to study the role of the carbohydrate in the functioning of a glycoprotein, the first step should be removal of all of the carbohydrate. If the protein remains active, the carbohydrate is not an essential part of the structure. If the activity is altered, the intact glycoprotein may be digested sequentially with purified glycosidases to determine which sugars are involved. Knowledge of the carbohydrate composition can be used to predict which glycosidases are most likely to be useful. At the same time, such experiments may provide evidence about the structure of the oligosaccharide chains (36). However,

structural studies are more informative when done on purified oligosaccharide chains. In cases where the carbohydrate has been shown not to be involved in biological activity, it is better to prepare glycopeptides or oligosaccharides for structural study.

The major difficulty in performing these experiments was the limited supply of epo. Only 70 μg (2 nmoles) of each form of epo was available for this study. The techniques described in the literature nearly all require milligram quantities of glycoprotein. Prior to actual experiments with epo it was necessary to develop a more sensitive gas-liquid chromatographic analysis for carbohydrate, a scaled-down procedure for hydrogen fluoride deglycosylation, and a method for separating free sugars from protein using 10- to 100-μl samples. These new procedures should be generally applicable to analysis of microgram quantities of glycoproteins.

CHAPTER II

MATERIALS AND METHODS

Analysis of Carbohydrate Composition

Analysis of carbohydrates by gas-liquid chromatography was developed from techniques used by Clamp, *et al.*, for separating the trimethylsilyl (TMS) ether derivatives (38) and by Zanetta, *et al.*, for separating the trifluoroacetate (TFA) ester derivatives (39). Prior to derivatization, samples were lyophilized in acid-cleaned 100-µl automatic sampler vials with Teflon-faced silicone septa. Either mannitol or inositol was added as an internal standard to every sample that was analyzed quantitatively. Samples were then refluxed in 100 µl of 0.5 M methanolic HCl for 12 to 16 hours at 80° to release methyl glycosides. When TFA esters were prepared, the methanolysates were dried *in vacuo* over P_2O_5 while still warm. In one experiment samples were neutralized by passing them over a 20 mg column of Amberlite IRA-400 (OH^-) twice. The columns were then rinsed with anhydrous methanol and the pooled effluents dried. When TMS ethers were prepared, the methanolysates were first neutralized with silver carbonate in slight excess, then reacetylated by adding 10 µl of acetic anhydride. This reaction was allowed to continue for 6 hours at room temperature. After reacetylation, the samples were centrifuged and the superna-

tant liquid transferred to a clean vial and dried *in vacuo*.

TMS ethers were formed by adding 10 to 20 µl of a solution of trimethylchlorosilane/hexamethyldisilazane/pyridine (0.2/0.4/1.0, v/v/v) to the dried methyl glycosides. After reacting for 15 minutes at room temperature, the sample was centrifuged to remove a white precipitate and 2 to 10 µl was injected onto the column. Separation of TMS ethers was performed by chromatography on 3% OV-1 columns with a temperature program beginning with a 10-minute isothermal period at 140° followed by a linear increase of 2° per minute to 215° and concluded with a 2-minute isothermal period at 215°. Nitrogen was used as a carrier gas at a flow rate of 30 ml per minute. The injectors were maintained at 260° and the detectors at 300°.

TFA esters were formed either from methyl glycosides prepared as described above or directly from monosaccharides. Nine µl of ethyl acetate and 1 µl of trifluoroacetic anhydride were added to a dried sample and the vial purged with nitrogen. The sample was then refluxed at 150° for 5 to 10 minutes and cooled in an ice bath. This step was repeated and 1 to 4 µl was injected onto the column. Separation of TFA esters was performed by chromatography on 5% SP-2401 columns with a temperature program increasing linearly at 1° per minute from 110° to 200°. Nitrogen was used as a carrier gas at a flow rate of 10 ml per minute. The injectors were maintained at 225° and the detectors at 300°.

Free neuraminic acid was measured by the thiobarbituric acid assay described by Warren (40) and modified by Hahn, *et al.* (41). Prior to analysis, oligosaccharides and proteins were hydrolysed in 0.1 N HCl at 80° for 1 hour or incubated with neuraminidase overnight at 37° in 0.1 M ammonium acetate, pH 5.0. β-Formylpyruvic acid released by periodate oxidation was quantitated by condensation with 2-thiobarbituric acid to produce a pink chromophore with a maximum absorbance at 549 nm. N-Acetylneuramin-lactose (NAN-lactose) was used as a secondary standard as a control for destruction of free N-acetylneuraminic acid during acid hydrolysis. The amount of N-acetylneuraminic acid in the NAN-lactose was determined by the thiobarbituric acid assay after digestion with soluble neuraminidase. Free N-acetylneuraminic acid was used as the standard.

Hydrogen Fluoride Deglycosylation

Hydrogen fluoride deglycosylation was carried out in 300-µl polypropylene microfuge tubes. The procedure was a modification of that described by Coudron, *et al.* (42). Samples were lyophilized, then dried further over P_2O_5 at 65° in vacuo. Immediately prior to deglycosylation, 1 µl of anisole and 10 µl of 70% hydrogen fluoride in pyridine were added for every 4 µg of protein. The reaction was allowed to continue for 4 to 24 hours at room temperature and was stopped either by drying the samples *in vacuo* under a NaOH trap or by drying them under a stream of dry nitrogen. Some

of the samples were dialyzed in a 100-µl microdialysis unit against deionized water or 0.1 M ammonium carbonate, pH 7.0, to remove fluorinated glycosides and any remaining anisole; others were used without further treatment.

Glycosidase Digestions

Individual or mixed glycosidases were incubated with samples in an appropriate buffer for 12 to 16 hours. Enzymes were added in 10 to 100 fold excess of the amount needed to completely digest the amount of sugar present in the time allotted. Controls containing only the sample and buffer were included in every experiment. Ammonium acetate, 0.1 M at various pH values, was used to buffer digestion mixtures containing purified glycosidases. In specified experiments 0.01 M calcium acetate was added to the buffer. In later experiments 50 µg/ml of gentamicin and 1 mg/ml of 4-times crystallized bovine serum albumin which was ethanol precipitated to remove minor contaminants were added to improve recovery of epo activity. The buffer used in digestion mixtures containing mixed glycosidases from *Streptococcus pneumoniae* was 0.05 M sodium cacodylate plus 1 mg/ml bovine serum albumin and 50 µg/ml gentamicin, pH 6.5.

Enzyme Assays

Glycosidases were assayed by monitoring the release of p-nitrophenol from the respective p-nitrophenylglycoside (43). Each enzyme was incubated with 200 µg of substrate for 15 minutes at 37° in 300 µl of the buffer in which the

enzyme would be used. The reaction was quenched by adding 2 ml of 1 M Na_2CO_3. Under these conditions, 0.001 unit of enzyme, defined as the amount needed to cleave 1 nmole of substrate in 1 minute, would produce an absorbance of 0.100 at 420 nm. Insoluble neuraminidase was assayed by incubating the enzyme with 40 µg of NAN-lactose for 2.5 minutes and analyzing 10 µl of the supernatant liquid for free neuraminic acid by the thiobarbituric acid assay.

Presence of protease was detected by the release of dye when a sample was incubated with 25 mg of Azocoll, a preparation of dye coupled to insoluble collagen, suspended in 2.5 ml of an appropriate buffer. To detect release of the dye, the tubes were centrifuged and the $A_{...}$ of the supernatant liquid recorded at intervals over 48 hours and compared to that of controls containing only buffer and Azocoll.

Separation of Free Sugar from Protein

Released sugars were separated from proteins after glycosidase digestion by ultrafiltration through Amicon YMB membranes in the Amicon MPS-1 micropartition system. Pressure for the filtration was applied by centrifuging the entire system at 1100 ×g. Membranes were rinsed free of glycerine by centrifuging 3 to 5 times with 1-ml aliquots of distilled water and the filtrate was discarded. Samples were then placed in the upper reservoir and centrifuged; the retentates were rinsed twice with distilled water. Pooled filtrates and retentates were analyzed for carbohydrate con-

tent by gas-liquid chromatography.

Determination of Apparent Molecular Weights

Fifteen percent SDS-polyacrylamide gels were prepared and run according to the method described by Laemmli (44). The gels were stained with Coomassie blue (45) or silver (46), or autoradiographed. Stained slab gels were phcto-graphed and either the gels themselves or the negatives were scanned in a Transidyne gel scanner. Tube gels containing ^{125}I-labelled samples were stained to locate the molecular weight markers and then cut into 1 mm slices and the radioactivity in the slices determined in an automatic gamma counter.

High pressure liquid chromatography (HPLC) was per-formed on a Toyasoda G-3000 molecular exclusion column using 0.15 M ammonium acetate, pH 7.1, as the buffer at a flow rate of 0.7 ml per min. All buffers were filtered through 0.45 μ filters before use. The A_{280} of the effluent was monitored and 0.25 ml fractions collected and the radioac-tivity measured. The column was calibrated with bovine serum albumin, ovalbumin, and cytochrome c.

Assays for Erythropoietin

The bone marrow assay was based on that described by Goldwasser, *et al.*, (18) with the following modifications. Bone marrow was flushed from the tibias and femurs of 5- to 10-week old rats and cultured in the chemically defined med-ium Syn-Med supplemented with 3 mg per ml of bovine serum

albumin, 5 μg per ml of bovine insulin, 5 μg per ml of human transferrin, 5 ng per ml of sodium selenate, and 50 μg per ml of gentamicin. No additional buffer or $Fe(NO_3)_3$ was added to the medium. No fetal calf serum supplement was used. The cells were plated at a concentration of 20×10^4 cells/ml. After 16 to 20 hours of incubation, 20 μl of 50% rat serum in Syn-Med containing 10 μCi per ml of $^{59}FeCl_3$ was added to each well. Five hours later the cells were collected into 13 × 100 mm tubes and lysed and washed with 5% trichloroacetic acid. The radioactivity in the pellets was measured in an automatic gamma counter to determine the amount of cellular protein-bound ^{59}Fe. Heme was released from the globin in the pellets and extracted into cyclohexanone. The radioactivity contained in 1-ml aliquots of the cyclohexanone was measured to determine the amount of heme synthesized during the five-hour pulse.

Radioimmunoassay was performed by Annette Gardner according to the procedure described previously (20). The antibodies used in these assays were prepared by Garcia (19) and by Sherwood and Goldwasser (20).

The polycythemic mouse assay was performed by Dr. Peter Dukes of Children's Hospital of Los Angeles (7). Polycythemia was induced by exposure to hypoxia for two weeks to suppress synthesis of erythropoietin by the mice and therefore decrease the baseline level of erythropoiesis. The mice were then injected with the samples on two consecutive days. On the third day they were injected with ^{59}Fe; on the fifth

day they were weighed and bled and the radioactivity in 1-ml aliquots of the blood was measured. The percent of ^{59}Fe incorporated into the total red cell mass was calculated using 8% of the body weight as the red cell mass for each animal. Six mice were used for each sample.

Statistical Methods

The mean and standard deviation were calculated for each group of replicates in an assay. The slope and intercept of the straight line approximating the standard curve of each assay were calculated using the linear regression program of a Hewett-Packard calculator. The statistical significance of differences between groups was established using Student's t test.

Sources of Materials

Source of Epo

The human urinary epo used in these experiments was purified by Miyake, *et al.* (2). Both the α- and β-epo fractions appeared homogeneous by polyacrylamide gel electrophoresis at pH 6 and pH 9 and SDS-polyacrylamide gel electrophoresis in 7.5% gels (2). Recent studies have shown that α- and β-epo have an identical, unique, N-terminal sequence (24). Portions of the α- and β-epo fractions were desalted by repetitive dilution and ultrafiltration. The amount of epo present in a sample was calculated from the A_{280} using a specific absorptivity of 8.5 for a 1% solution

(2). The value for the molecular weight of epo used in all calculations is the recently revised estimate of 34,000 (47). [^{125}I]iodoepo was prepared from purified α-epo using the Iodo-Gen method (20). Purified human serum α_1-acid glycoprotein was a gift from Dr. Karl Schmid, Boston University.

Materials used in Carbohydrate Analysis

Mannosamine,[1] glucoheptose, mannoheptulose, sedoheptulose anhydride monohydrate, 2-deoxyglucose, rhamnose, fructose, sorbose, L-amino acids, N-acetylneuramin-lactose, grade I, and both soluble and immobilized type X neuraminidase from *Clostridium perfringens* (EC 3.2.1.18) were purchased from Sigma Chemical Co., St. Louis, MO. Other monosaccharides, 0.5 N methanolic HCl, and 4 mm i.d. 6-foot glass columns packed with either 3% OV-1 methyl silicone adsorbed on 100/120 mesh Chromosorb WHP or 5% SP-2401 trifluoropropylsilicone adsorbed on 100/120 mesh Supelcoport were obtained from Supelco, Inc., Bellefonte, PA. Ultra-high-purity grade nitrogen and hydrogen and hydrocarbon-free grade air were purchased from Linde Division Union Carbide Corp., New York, NY. Trifluoroacetic anhydride, hexamethyldisilazane, trimethylchlorosilane, Sequanal-grade ethyl acetate, and silylation-grade pyridine were obtained

[1]All sugars used in this study had the D configuration except fucose and sorbose, which had the L configuration.

from Pierce Chemical Co., Rockford, IL. Amberlite IRA-400 (OH^-) was obtained from Fisher Scientific Co., St. Louis, MO. 2-Thiobarbituric acid was purchased from Eastman Kodak Co., Rochester, NY.

Gas-liquid chromatography was carried out in a Perkin Elmer Sigma 2 Gas Chromatograph equipped with dual flash vaporizing injectors and flame ionization detectors. All analyses were made using a matched compensating column to subtract that portion of the signal due to column bleed. Peak identification and integration were carried out by a Perkin Elmer Sigma 10 Data Processing Station programmed to construct an appropriate baseline.

Materials used to Prepare Deglycosylated Epo

Purified β-galactosidase (EC 3.2.1.23) and β-hexosaminidase (EC 3.2.1.52) from the liver of *Charonia lampus* were purchased from Miles Laboratories, Inc., Elkhart, IN., along with purified *S. pneumoniae* endoglycosidase D (EC 3.2.1.96) and *Streptococcus griseus* endoglycosidase H (EC 3.2.1.96). Protease-free mixed glycosidases from *S. pneumoniae* were the gift of Dr. Robert Hill, Duke University and were assayed in his laboratory (48). Four-times-crystallized bovine serum albumin was purchased from ICN Nutritional Biochemicals, Cleveland, OH.

Seventy percent hydrogen fluoride dissolved in pyridine was purchased from Columbia Organic Chemicals, Columbia, SC. Anisole was purchased from Aldrich Chemical Co., Milwaukee,

WI. P-nitrophenylglycosides were purchased from Sigma Chemical Co., St. Louis, MO. Azocoll was obtained from Calbiochem-Behring, San Diego, CA.

Materials used in Separations Based on Molecular Weight.

Electrophoresis-grade acrylamide, bis-acrylamide, sodium dodecylsulfate (SDS), and glycine, along with molecular weight standards for SDS-polyacrylamide gel electrophoresis, were purchased from Bio-Rad Laboratories, Richmond, CA. ^{14}C-methylated molecular weight standards were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, MD. Purified proteins used as molecular weight standards in molecular exclusion chromatography were obtained from Mann Research Laboratories, Orangeburg, NY. The Amicon MPS-1 micropartition system was purchased from Amicon Corp., Danvers, MA., along with 14-mm YMB membranes. High pressure liquid chromatography (HPLC) was performed on a Toyasoda G-3000 molecular exclusion column from Varian Associates, Inc., Walnut Creek, CA. The effluent was monitored with a Bio-Rad flow-through UV monitor model 1305.

Materials used in Assays of Epo

Rat serum was obtained from Pel-Freeze Biologicals, Rogers, AR. SynMed medium was purchased from Centaurus Biological Corp., Anaheim, CA., and Insulin/Transferrin/Selenium supplement from Collaborative Research, Waltham, MA. Gentamicin was purchased from Schering Corp.,

Kenilworth, NJ. $^{59}FeCl_3$ was obtained from Amersham Corp., Arlington Heights, IL.

CHAPTER III

RESULTS

Determination of Carbohydrate Composition

Development of Gas-Liquid Chromatographic Analysis

TMS ethers and TFA esters of methanolyzed samples containing fucose, galactose, mannose, glucose, N-acetylglucosamine, N-acetylgalactosamine, N-acetylneuraminic acid, mannitol, and inositol, singly and in mixtures, were analyzed by gas-liquid chromatography. The pure monosaccharides which were used are those commonly found in mammalian glycoproteins (49). The alditols were chosen as internal standards because they are not constituents of glycoproteins. Each monosaccharide produced several peaks representing the different anomers present. The relative retention times and relative proportion of the peaks produced by each sugar are shown in Table 1 for the TMS ethers and in Table 2 for the TFA esters. The patterns of peaks in the chromatograms are in good agreement with those described by Clamp, *et al.*, (38) and Zanetta, *et al.*, (39), respectively. Those peaks whose area was used in calculating the recovery of sugar are marked with an asterisk in Table 2.

Separation of the TFA esters of the hexoses and N-acetylhexosamines is superior to separation of the TMS ethers

of those sugars. In analyses of low concentrations of monosaccharides using the TMS ether derivatives, galactose 1 appears as a shoulder on the major mannose peak and mannose 2 as a shoulder on the major galactose peak. The separations between the overlapping pairs are 0.57 and 0.76 minutes, respectively. In contrast, the two closest hexose peaks in the chromatogram of the TFA esters are separated by 1.09 minutes. A major peak of the TMS ether of glucosamine overlaps the second peak of galactose. The major peak of the TFA ester of glucosamine appears 9 minutes later than the hexoses. The unacetylated hexosamines are not normal constituents of glycoproteins (49), but may occur in a sample as a result of deacetylation during derivatization (50). Deacetylation during formation of the TFA esters, however, normally affects less than 10% of the sample (39), so that the minor peaks of the hexosamines represent negligible contaminants in the hexose region. Since the major hexosamine peaks are well separated from both the hexoses and the major peaks of the N-acetylhexosamines, it is possible to monitor the degree of deacetylation to detect any interference that may occur. This is not possible with the TMS ethers. The behavior of the internal standard was also a problem when the TMS ethers were analyzed. Two peaks were often obtained for the TMS ether of mannitol where only one was predicted (38). The area of the second peak ranged from 0 to 20% of the total area of the mannitol peaks. For these reasons, the TFA esters were used in all subsequent analyses.

Analysis of the TFA esters of methyl glycosides offers increased recovery as well as improved separation. Recovery may be expressed as the area of a peak[1] divided by the amount of the corresponding sugar injected. Peaks smaller than 0.5 area units cannot be reliably distinguished from the baseline. Even when small peaks are detected, the area cannot be measured precisely because small fluctuations in the baseline are of the same magnitude as the peak area itself. Peaks should contain at least 5 to 10 area units for quantitation. The TMS ethers produced 3.7 ±1.7 area units per μg of inositol while the TFA esters produced 190 ±74 area units per μg of inositol, a 50-fold increase in recovery.

The recovery varies considerably from one analysis to another depending on the proportion of the sample lost during preparation. These losses affect the entire sample uniformly and are compensated for by the internal standard included in every quantitative analysis. However, such variation means that some analyses of very small samples will be unsuccessful because too little sugar will be recovered to quantitate or detect. The recovery can be used to calculate the minimum amount of sugar which can be detected or quantitated routinely. Assuming that the variation in recovery is described by a normal distribution, 84% of the

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[1]Area is expressed in the arbitrary area units reported by the Sigma 10 Data Station.

analyses will have a recovery at least as large as the mean recovery minus one standard deviation; 98% of the analyses will have a recovery at least as large as the mean recovery minus two standard deviations. Thus, 84% of the analyses using the TFA ester would be able to detect 4 ng of inositol and 98% would be able to detect 11 ng of inositol. To ensure a 98% probability of obtaining a usable quantitative analysis, as opposed to merely detecting the presence of sugar, one hundred to two hundred ng of inositol would be needed. A correspondingly larger mass would be needed for those sugars which produce more than one peak.

As a result of minor differences in carrier gas flow and injection timing, the absolute retention time of a peak varied by 1% to 2% and the relative retention time by less than 0.5% among sequential analyses. However, the absolute retention time of inositol varied by as much as 17% when analyses made on different columns were compared. These variations resulted from compression or expansion of the entire chromatogram. The pattern and order of elution of the sugars was not affected. The percent variation in absolute retention time was therefore largest for those sugars which eluted earliest. Variations in relative retention time did not exceed 4.0% and were largest for sugars which eluted furthest from the internal standard, increasing as the absolute retention time of the standard decreased. For this reason, peak identification was made using relative retention times.

To determine whether or not neutralization of methanolysates is necessary, samples of mixed monosaccharides were either dried *in vacuo* immediately after methanolysis or first neutralized by passage through a column of Amberlite IRA-400 (OH^-) resin. The TFA esters of the samples were chromatographed and total recoveries and relative response factors were compared for each sugar. Unlike the results obtained by others (50,51), the recovery of each sugar was higher in the samples dried *in vacuo* immediately than in those first neutralized on the ion exchange resin. The molar response factors (defined in the Appendix) of galactose, mannose, N-acetylglucosamine, and N-acetylneuraminic acid were smaller for the neutralized samples than for the samples dried immediately, indicating loss of these sugars relative to inositol in the neutralized samples. The response factors of fucose, glucose, and N-acetylgalactosamine were larger. The fucose and N-acetylgalactosamine peaks were misshapen, suggesting that they overlapped contaminant peaks. The excess glucose may have been a contaminant in the resin which eluted into the samples. Several large unexplained peaks were also noted in the chromatogram of the neutralized samples and the baseline was irregular. No benefit was observed in neutralizing the methanolysates before drying them.

The retention times of methanolyzed samples of a number of sugars not commonly found in glycoproteins were examined to be sure that they could be identified if present. The

results are shown in Table 3. All of the sugars examined except glucoheptose were well separated from the sugars normally found in glycoproteins. The first peak of glucoheptose overlapped with mannose 2, while the second peak fell in a region where an uncharacterized contaminant regularly appeared. In this case the relative proportions of the peaks were used to rule out the presence of glucoheptose. The ketoses sorbose and fructose produced no peaks whatsoever during the period of the chromatogram. A sample purchased as 90% N-glycolylneuraminic acid was indistinguishable from N-acetylneuraminic acid, although Zanetta, et *al.*, reported that the main peak for N-glycolylneuraminic acid was retained approximately six minutes longer than that of N-acetylneuraminic acid (39).

A number of non-carbohydrate substances which produce peaks in the chromatogram are listed in Table 4 along with their retention times. Many of these chemicals are common buffer components. Others are used in the manipulation of proteins. All except Tris-hydroxymethylaminomethane (THAM) interfere with the carbohydrate analysis. These substances should not be used with samples intended for carbohydrate analysis. Free amino acids also interfere with the analysis, producing peaks in all regions of the chromatogram (data not shown). However, methanolysis under the conditions used here does not release amino acids from proteins. Bovine serum albumin, which is not glycosylated, produced no major peaks after methanolysis and could be freed from minor

contaminants by further precipitation of 4-times-crystallized albumin with ethanol.

Analysis of Erythropoietin

A preliminary analysis of purified epo showed the presence of fucose, galactose, mannose, N-acetylglucosamine, N-acetylneuraminic acid, THAM, traces of glucose, and possibly traces of N-acetylgalactosamine. Based on these results, an external standard of pure monosaccharides mixed in the approximate proportions found in epo was prepared. The response factors calculated from 8 analyses of this standard are shown in Table 5. The standard mixture was analyzed before and after each analysis of an epo sample. Analysis of this standard on a fresh pair of columns demonstrated that small amounts of N-acetylgalactosamine could be separated from the minor peak of large amounts of N-acetylglucosamine, as shown in Figure 1.

The carbohydrate content of three samples containing 11.8 μg (0.347 nmoles) of α-epo and three samples containing 14.8 μg (0.435 nmoles) of β-epo was analyzed. All of the samples except one α-epo sample had been desalted. The results are reported in Table 6. One of the chromatograms is shown in Figure 2. The results confirm the presence of fucose, galactose, mannose, N-acetylglucosamine, and N-acetylneuraminic acid and rule out the presence of N-acetylgalactosamine. Small traces of glucose remain in the desalted samples but over 99% of the THAM has been removed. As shown

in the top half of Table 6, there was a statistically significant difference in the N-acetylglucosamine and N-acetylneuraminic acid content of α-epo and β-epo, with the α-epo containing more total carbohydrate.

Independent Sialic Acid Analysis

Since quantitation of N-acetylneuraminic acid by gas-liquid chromatography is relatively imprecise, the neuraminic acid content of epo was also measured by an independent method. The thiobarbituric acid assay can measure as little as 25 ng of free neuraminic acid (41). Sialic acids may be released by either acid hydrolysis or neuraminidase treatment. A sample of purified human serum α_1-acid glycoprotein released 7.3 moles of neuraminic acid per mole of protein after treatment with 0.05 units of immobilized neuraminidase for 4 hours. After further hydrolysis in 0.1 N HCl for 1 hour, the same sample contained a total of 11.7 moles of free neuraminic acid per mole of protein, indicating that the neuraminidase treatment had released less than 60% of the neuraminic acid attached to human serum α_1-acid glycoprotein. Isemura and Schmid report that human serum α_1-acid glycoprotein contains 14 moles of neuraminic acid per mole of protein (52). Degradation of sialic acids occurs during acid hydrolysis (53). Since free neuraminic acid might be more rapidly degraded than glycosidically linked neuraminic acid, NAN-lactose was used as a secondary standard. The purity of the NAN-lactose was determined by the

thiobarbituric acid assay after hydrolysis with soluble neuraminidase. No free neuraminic acid was detected in the thiobarbituric acid assay of unhydrolyzed NAN-lactose. Digestion of 1 to 3 μg of NAN-lactose with 10 milliunits of soluble neuraminidase for 14 hours released an average of 410 ng of N-acetylneuraminic acid per μg of sample. Since NAN-lactose contains 1 mole of N-acetylneuraminic acid per mole of trisaccharide, the NAN-lactose is 84% pure. After digestion with 0.1 M HCl at 80°, only 320 ng of N-acetylneuraminic acid were recovered per μg of sample, indicating that 20% of the N-acetylneuraminic acid was lost.

The results of analysis of three 1.22 μg (35.8 pmole) samples of α-epo and three 1.26 μg (36.9 pmole) samples of β epo are shown in Table 7. The values obtained by thiobarbituric acid analysis were not significantly different from those obtained by gas-liquid chromatographic analysis. The thiobarbituric acid analysis confirms that α-epo contains more N-acetylneuraminic acid than β-epo, and that the difference is statistically significant. The difference in N-acetylneuraminic acid content between the two forms does not account for the difference in mobilities at pH 9. In fact, if the different neuraminic acid content were causing the variation in mobility, α-epo would exhibit a higher mobility than β-epo.

Hydrogen Fluoride Deglycosylation

Anhydrous hydrogen fluoride, either as a pure liquid or dissolved in pyridine, can be used to deglycosylate proteins. Preliminary experiments using human serum α_1-acid glycoprotein as a model demonstated that the technique of hydrogen fluoride deglycosylation described by Coudron, *et al.* (42) could be scaled down 500-fold while retaining 95% recovery of the deglycosylated protein by evaporating the hydrogen fluoride and pyridine in a 200 μtorr vacuum under a NaOH trap instead of precipitating the protein. Decreased recoveries were observed when samples were dried under a stream of dry nitrogen instead of *in vacuo*. Later experiments showed that dialysis to remove the fluorinated glycosidic byproducts of the reaction was unnecessary because they did not interfere with SDS-polyacrylamide gel electrophoresis, carbohydrate analysis, or the *in vitro* bone marrow assay.

Most glycoproteins are completely deglycosylated in 1 to 3 hours of treatment with hydrogen fluoride in pyridine (54). However, samples of human serum α_1-acid glycoprotein deglycosylated for varying periods continued to decrease in apparent molecular weight during 16 hours of reaction, after which they remained constant at an apparent molecular weight of 24,000. Because of this variation, epo samples were treated for a variety of times.

Samples containing 2.8 or 5.0 μg (0.082 or 0.147 nmoles) of β-epo or 130,000 cpm of [^{125}I]iodoepo were

deglycosylated by treatment with hydrogen fluoride in pyridine for 6 to 8 hours. One 2.8 μg (0.082 nmole) sample was treated for 19 hours. The 2.8 μg samples were dried under nitrogen and then dialyzed; the other samples were dried *in vacuo* and analyzed without dialysis. Eighty percent of the 5 μg sample was subjected to carbohydrate analysis. The resulting chromatogram is shown in Figure 3. The peaks seen in the region between 5 minutes and 20 minutes are found only in hydrogen-fluoride-treated samples that have not been dialyzed. Presumably they represent the fluorinated glycosidic byproducts described by Mort and Lamport (37). The only sugar present in quantity is glucose, which was not a component of the original sample. Small peaks corresponding to galactose, mannose, N-acetylglucosamine, and N-acetylneuraminic acid are present. The recovery of each sugar is estimated in Table 8. Over 75% of the carbohydrate has been removed.

On SDS-polyacrylamide gels the samples treated for 6 to 8 hours migrated as a diffuse band with an apparent molecular weight range of 35,000 to 29,000, but with a significant tail extending to the dye front. A sample treated for 6 hours is shown in lane 3 of Figure 4. No protein was detectable in the 19-hour sample.

The recovery of *in vitro* biological activity in samples deglycosylated for 6 to 8 hours was approximately 1%. Only 0.1% of the immunological activity of those samples was recovered. Neither biological nor immunological activity

was recovered from the sample deglycosylated for 19 hours. The loss of biological and immunological activity after hydrogen fluoride treatment is most likely the result of degradation of the polypeptide chain. Epo activity is very sensitive to proteolysis (3). Therefore, hydrogen fluoride deglycosylation cannot answer questions about the importance of the carbohydrate portion of epo to its activity.

Deglycosylation by Glycosidase Treatment

Monitoring Glycosidase Reactions

Glycosidases can remove the carbohydrate from a glycoprotein without affecting the polypeptide. However, glycosidases are also protein preparations and can introduce contaminants into the sample. All of the glycosidases used in this study had such low specific activities that the amount of protein in a 0.005 unit aliquot was as large as the amount of epo in the sample. The difficulties introduced by such contamination can be minimized by using purified glycosidase preparations containing no carbohydrate. In addition, the glycosidases release unmodified monosaccharides. To measure monosaccharides in the presence of oligosaccharides either the two must be separated or the free sugars must be analyzed without formation of the methyl glycosides. Methanolysis would cleave the oligosaccharides to methyl glycosides at the same time that the methyl glycosides of the monosaccharides are being formed.

Precipitation of the glycoprotein should leave the free sugar in the supernatant liquid, which could then be analyzed separately from the pellet. Unfortunately, as shown in Table 4, both trichloroacetic acid and phosphotungstic acid dissolved in 0.1 N HCl, the most effective precipitating agents, produce peaks which interfere with the analysis. Other precipitating agents such as ethanol do not completely precipitate microgram quantities of glycoproteins.

An attempt was made to analyze free monosaccharides in the presence of oligosaccharide material. The retention times of the TFA esters of monosaccharides are shown in Table 9. Except for galactose and glucose, the peaks are well separated. The minor peaks of galactose may be used to distinguish between galactose and glucose. However, when unmethanolyzed mixtures of proteins and free sugars were analyzed repeatedly, the recovery of sugar declined markedly and the baseline of the chromatogram began to deteriorate.

The Amicon MPS-1 micro-ultrafiltration system was tested to see whether it could separate high and low molecular weight species without adding contaminants that would interfere with the analysis. Samples containing 60 μg of carbohydrate-free bovine serum albumin, 750 μg of ovalbumin, or a mixture of 1 μg of each monosaccharide were filtered in the MPS-1 system and the carbohydrate composition of both the filtrates and the retentates determined by gas-liquid chromatography. The results are shown in Table 10.

The filtrates of the carbohydrate-free bovine serum

albumin and intact ovalbumin produced few peaks in the chromatogram. The major peak, with a relative retention time of 1.87, did not correspond to any carbohydrate peak. The filtrate of the carbohydrate-free bovine serum albumin did contain small peaks with relative retention times corresponding to fucose and mannose, and a larger pair of peaks with retention times corresponding to glucose. The filtrate from the mixture of monosaccharides contained the expected amounts of mannose, galactose, and N-acetylglucosamine, more fucose and N-acetylgalactosamine than expected, and less N-acetylneuraminic acid than expected. The recovery of protein in the retentate is not quantitative; of 750 μg in the original ovalbumin sample, approximately 250 μg could be collected from the upper reservoir after micro-ultrafiltration. All of the retentates contained significant quantities of glucose and contaminants eluting with the hexosamines. However, the retentates of the monosaccharides and carbohydrate-free bovine serum albumin contained only traces of galactose and mannose and no fucose.

Thus, monosaccharides can be separated from glycoproteins by the Amicon MPS-1 ultrafiltration system. By analyzing both the filtrate and the retentate from a sample, it is possible to estimate the release of those sugars found in epo and glycoproteins of similar composition. The analysis would not be useful for following the release of glucose or N-acetylgalactosamine.

This technique may also be used to confirm that

glycosidases are active. The activity of exoglycosidases can be checked easily by using p-nitrophenyl glycosides. No similar substrates are commercially available for use with endoglycosidases. Endoglycosidase H will remove the high-mannose N-linked chains found in ovalbumin (36). Endoglycosidase D should remove the core tetrasaccharide of complex N-linked chains of human serum α_1-acid glycoprotein, but only after the external N-acetylneuraminic acid, galactose, and N-acetylglucosamine have been removed (36,55). The exoglycosidases remove only terminal sugars.

Samples of ovalbumin and α_1 acid glycoprotein were incubated for 24 hours with endoglycosidase H or a mixture of neuraminidase, β-galactosidase, β-hexosaminidase, and endoglycosidase D, respectively. The released sugars were separated from the proteins by micro-ultrafiltration and both filtrates and retentates analyzed by gas-liquid chromatography. No significant peaks assignable to sugars were found in the filtrates, implying that the exoglycosidases and the endoglycosidase H were not active on human serum α_1-acid glycoprotein and ovalbumin, respectively. The retentates contained all of the sugars normally found in ovalbumin and α_1 acid glycoprotein except N-acetylneuraminic acid, confirming that neither the β-galactosidase nor the endoglycosidase H was active. The failure of the released N-acetylneuraminic acid to appear in either the filtrate or the retentate is unexplained. All of the exoglycosidases used had been active on low molecular weight substrates. A

number of glycosidases can not digest oligosaccharides linked to protein (36). Since the purified glycosidases were not active on intact glycoproteins, it was necessary to use the crude preparation of mixed glycosidases from *S. pneumoniae* to deglycosylate epo.

Choice of Buffers for Glycosidase Reactions

A series of experiments was performed to devise a system in which the release of sugars by glycosidases and resulting alterations in biological acitvity could both be measured on the same sample of epo. For maximum sensitivity, samples for gas-liquid chromatography should not contain large amounts of inorganic salts (56) or any of the buffers which interfere with the analysis (see Table 4). However, the glycosidase digestion mixture must be buffered. Volatile buffers such as ammonium acetate or ammonium bicarbonate can be removed from the sample by lyophilization prior to gas-liquid chromatography. Ammonium acetate, 0.1 M, containing 1 mg per ml of ethanol-precipitated, 4-times-crystallized bovine serum albumin and 50 μg per ml of gentamicin did not produce any peaks which would interfere with the carbohydrate analysis. However, the presence of 5 mM ammonium acetate (equivalent to the addition of 10 μl of undiluted digestion mixture per culture) in the *in vitro* bone marrow cultures caused a 57% suppression of heme synthesis. Addition of 0.005 units of epo along with the ammonium acetate elicited only a 47% stimulation of heme

synthesis, while in the absence of ammonium acetate, 0.005 units of epo elicited a 70% stimulation of heme synthesis. The amount of heme synthesized in the presence of 0.005 units of epo and 5×10^{-3} M ammonium acetate was less than the amount of heme synthesized in the absence of any additions. This suppression of the bone marrow assay could be avoided by diluting the samples, since 0.05 mM ammonium acetate had no detectable effect on the bone marrow assay.

Analysis of sugars released by the *S. pneumoniae* mixed glycosidases is pointless because the glycosidase mixture is a relatively crude preparation containing 100 µg of protein, 175 ng of galactose, 230 ng of glucose, 260 ng of N-acetylglucosamine, and 120 ng of N-acetylgalactosamine per 5 µl aliquot, 5- to 10-fold more than the largest epo sample studied. Under these conditions there is no direct way to confirm that the epo has been deglycosylated. As a result, the buffer used in digestions with *S. pneumoniae* glycosidases does not need to meet the stringent requirements for gas-liquid chromatographic analysis. Therefore, digestions with these glycosidases were done in the cacodylate buffer recommended by Glasgow, et *al.*, (48).

The presence of a 1:2500 dilution of the *S. pneumoniae* glycosidases and 2.5 mM cacodylate buffer in the *in vitro* bone marrow assay cultures produced a 74% suppression of heme synthesis. Presence of a 1:250,000 dilution of the glycosidases and 0.025 mM cacodylate buffer had no detectable effect on heme synthesis, but 0.025 mM cacodylate buffer

alone caused a 13% increase in heme synthesis that was significantly different from the baseline at the P = 0.05 level, suggesting that even a 1:250,000 dilution of the enzymes might supress heme synthesis slightly. For assay of digestion mixtures containing the *S. pneumoniae* glycosidases, cultures were supplemented with buffer and enzymes so that each culture contained the same concentration of both substances. Aliquots of cacodylate buffer and mixed glycosidases were added to the control wells in subsequent assays so that all wells had an equal concentration of buffer and enzymes.

Enzymatic Deglycosylation of Epo

Samples containing 0.35 to 62 units (0.147 to 26.0 pmoles) of native epo or 5000 to 45,000 cpm of [^{125}I]iodoepo were incubated with *S. pneumoniae* mixed glycosidases for times ranging from 2 minutes to 21 hours. The samples of [^{125}I]iodoepo were analyzed by SDS-polyacrylamide gel electrophoresis and molecular exclusion chromatography to demonstrate changes in the apparent molecular weight of epo treated with the glycosidases.

By SDS-polyacrylamide gel electrophoresis, the apparent molecular weight of epo is decreased by 27% after treatment with the *S. pneumoniae* glycosidases. As shown in Figures 4 and 5, the glycosidase-treated samples contain no residual intact epo. The minor band migrating more rapidly than epo in Figure 4 is found in both untreated and glyco-

sidase-treated samples. It is most likely a radiolytic degradation product. As seen in Figure 4, there is little further decrease in apparent molecular weight after two minutes of treatment with glycosidases, indicating that the reaction is very rapid.

A slab gel may be distorted by drying it for autoradiography. Distortion of the gel results in incorrect measurment of the mobilities of the proteins. To avoid this source of error, samples of [^{125}I]iodoepo were incubated for 14 hours in the presence or absence of the *S. pneumoniae* glycosidases and electrophoresed in 15% SDS-polyacrylamide tube gels. Figure 5 shows the distribution of radioactivity in these gels. The apparent molecular weight of the untreated epo peak is 39,000; that of the glycosidase-treated epo peak is 28,500. This difference represents a loss of 10,500 daltons, or 27% of the apparent molecular weight of epo. The carbohydrate composition determined above shows that epo contains approximately 11,000 daltons of carbohydrate. Therefore, loss of 10,500 daltons due to deglycosylation would represent removal of 95% of the carbohydrate. One hundred percent of the radioactivity in the glycosidase-treated sample was recovered from the gel. Only 30% of the radioactivity was recovered in the gel containing undigested epo because fully glycosylated epo is poorly fixed by acetic acid/isopropanol fixative (2). The complete recovery of radioactivity from the glycosidase-treated sample coupled with the lack of any low molecular weight bands

of radioactivity makes it very unlikely that proteolytic degradation caused the decrease in molecular weight.

As shown in Figure 6A, the HPLC chromatogram of untreated [^{125}I]iodoepo contains three peaks: a small peak, A, at the void volume which is an aggregate of epo, the monomer peak, B, with an apparent molecular weight of 53,000, and a peak C with an apparent molecular weight of 7,300 which is believed to be a radiolytic degradation product. The only changes in this chromatogram upon incubating the epo at pH 6.5 and 37° for up to 21 hours are that the apparent molecular weight of peak B shifts gradually to 39,000 and that the ratio of the radioactivity in peak A to that in peak B gradually increases from 0.2 to 1.0. The molecular weight of peaks B and C at various times of incubation is shown in Table 11. The iodoepo preparation used for the 15 minute incubation was two weeks older than that used for the other incubations. This aging may account for the lower molecular weight of peak C in that experiment.

Heavily glycosylated proteins are known to behave anomalously on molecular exclusion chromatography (26). The apparent molecular weight obtained by this method is heavily dependent on the Stokes' radius of the protein. The decrease in the apparent molecular weight of epo seen after incubation at 37° in 0.05 M cacodylate buffer, pH 6.5, may reflect a change in the conformation of the protein. The apparent molecular weight obtained by SDS-polyacrylamide gel electrophoresis does not change after incubation for up to