EXHIBIT E
PART 2

14 hours.

The appearance of the HPLC chromatogram of glycosidase-treated samples of [^{125}I]iodoepo depends on the length of time the samples were incubated with enzyme. As shown in Figure 6A and Table 11, after two minutes of incubation at 20° the apparent molecular weight of the monomer has decreased by 34%. The proportion of the total radioactivity in each peak is the same as for untreated epo.

Figures 6B to 6D show the effects of incubating epo with the glycosidases for 15 minutes or 6 or 21 hours. The ratio of radioactivity in peak A to that in peak B is as much as 25 times the ratio seen with untreated epo, indicating that glycosidase-treated epo is much more likely to aggregate than native epo.

A fourth peak, D, of very low molecular weight, appears in the HPLC chromatogram of samples incubated with glycosidases for 6 or 21 hours (see Figures 6C and 6D). The identity of this peak is unknown. If it were the deglycosylated analog of peak C, it should appear in the chromatogram after shorter incubations.

In Table 12 the immunological and *in vitro* biological activities of glycosidase-treated epo are compared with the activities of epo incubated in the absence of enzymes. The enzyme-treated epo retained an average of 60% of the control activity in 4 preparations. The immunoactivity is consistently lower than the *in vitro* activity, averaging 40% of the activity of the control in 3 preparations. During the

incubation the control loses 25% to 35% of its original activity. The *in vitro* activity recovered after deglycosylation is therefore 35% to 45% of that present in the original sample. However, the HPLC studies show that as much as 60% of the glycosidase-treated epo is aggregated. The aggregate of native epo is inactive (57). If, as is likely, the aggregate of the enzyme-treated epo is also inactive, the specific activity of the residual monomer would be 4 to 6 times that of native epo. Samples submitted to an *in vivo* polycythemic mouse assay performed by Dr. Peter Dukes retained less than 1% of their activity.

CHAPTER IV

DISCUSSION

Development of Techniques

Carbohydrate Analyses

Analysis of carbohydrate polymers by gas liquid chromatography requires that they first be cleaved to monosaccharides. Aqueous acid, glycosidic enzymes, and anhydrous methanolic HCl will all release sugars from glycoproteins. However, acid hydrolysis vigorous enough to remove all sugar residues from the protein results in extensive destruction of the free reducing sugars (50). Glycosidic enzymes may themselves be glycoproteins, contaminating the sample (58,59). In addition, the enzymes have stringent substrate specificities and, even in combination, may not completely degrade the oligosaccharide (36). Methanolysis is preferable because it produces stable methyl glycosides that are more cleanly separated by gas-liquid chromatography than the analogous monosaccharides (39,60). Chambers and Clamp show that treatment with 1 M HCl in anhydrous methanol at 85° for three hours completely released the sugars from a variety of glycopeptides and a mucus glycoprotein. Recovery of sugar did not decrease when methanolysis continued for 24 hours (50). Other authors suggested that 0.5 M methanolic HCl,

commercially available, would be adequate (61,62).

The methanolic HCl is evaporated prior to further derivatization. Degradation of the sugars has been reported when samples were not neutralized before being dried (50,51). In contrast, we obtain a lower recovery of sugar in samples neutralized by passage through an ion-exchange column as suggested by Rickert and Sweeley (51) than in samples dried immediately after methanolysis. Previous authors have dried their methanolysates under a stream of dry nitrogen, while in this study the samples were dried *in vacuo*. A similar effect of increased degradation in samples dried under nitrogen compared with samples dried *in vacuo* is seen in samples deglycosylated with hydrogen fluoride dissolved in pyridine.

Neither methyl glycosides nor monosaccharides are sufficiently volatile to permit separation by gas-liquid chromatography without derivatizing them. Three derivatives have been proposed that would permit simultaneous analysis of the sugars normally found in glycoproteins. Both TMS ethers and TFA esters of relevant methyl glycosides can be separated at temperatures lower than 215° in 50 to 90 minutes (38,39). The major drawback with these derivatives is that each monosaccharide produces separate peaks corresponding to the pyranose and furanose anomers (39,50). The hexose O-methyloximes and hexosamine aldononitrile acetates yield single peaks (63). Unfortunately, several additional steps are required to prepare the oximes and aldonoditrile

acetates, including cleavage of the methyl glycosides and separation of neutral and amino sugars. These procedures are not practical with samples in the nanomolar range.

The TMS derivatives have been used extensively by investigators characterizing glycoproteins (52,64-72). However, several problems hinder analysis of very small amounts of sugar by this technique. The most serious of these involve loss of methyl glycosides and alditols during manipulations of the methanolysates prior to derivatization.

N-acetylhexosamines may undergo partial deacetylation during methanolysis (50,51,61). Because the TMS derivatives of β-methylgalactosaminide and α-methylmannoside have the same retention time, as do those of β-methylglucosaminide and α-methylgalactoside, it is necessary to reacetylate samples containing mixtures of these sugars (50). N-acetylation without significant O-acetylation is achieved with acetic anhydride in the presence of Ag^{+} (73,74). Many investigators have used silver carbonate, thus neutralizing the methanolysate and providing Ag^{+} in a single step (38). However, the resulting silver chloride forms an insoluble complex with methyl glycosides (50,51). This phenomenon is probably responsible for the low sensitivity of the analysis using the TMS ethers in the current study. At low concentrations of sugar, the loss of alditols normally used as internal standards greatly exceeds that of α-methylglucoside (51). Failure of the internal standard to behave the same way as the rest of the sample can invalidate the analysis.

The results of the current study also show that when small samples of TMS-derivatized sugars are analyzed the hexoses are not completely separated.

Trifluoroacetic anhydride was first suggested as a volatizing agent for sugars by Arakawa, Imanari, and Tamura (75,76) and developed for use with methyl glycosides by Zanetta, *et al.*, (39) and Tomana, *et al.*, (56). Eklund, *et al.*, adapted these procedures for use with capillary columns and electron capture detectors (77). The derivatization has been carried out in tetrahydrofuran at 0° (76), in dichloromethane at 130° to 150° (39,77), and in ethyl acetate at room temperature (56,75). Tomana, *et al.*, report that ethyl acetate is the preferred solvent (56). The reaction proceeds more rapidly at elevated temperature and the procedure described in Chapter 2 provides simple, rapid, and efficient derivatization. Because the reagent is vaporized and recondensed, portions of the sample splattered on the sides of the vial during drying are recovered. The separation achieved by Zanetta, *et al.*, using a trifluoropropylsilicone stationary phase was superior to that obtained by Tomana, *et al.*, on a cyanopropylmethylsilicone stationary phase (39,56) and the former phase was chosen for this study.

The problems associated with the analysis of TMS ethers may be avoided by using the TFA esters. Reacetylation is not necessary when TFA derivatives are used because the major peaks of the hexosamines are completely separated from

the hexoses. In agreement with the results obtained by Zanetta, *et al.*, (39), deacetylation of hexosamines was normally less than 10%. In general, separation of the TFA esters is superior to that of the TMS ethers. In addition, analysis of TFA esters is 50-fold more sensitive than analysis of TMS ethers. With the TFA esters it is possible to detect 5 to 10 ng of an alditol and 10 to 20 ng of a methyl glycoside.

In fact, the major limitation to sensitivity in the analysis of TFA esters is chemical rather than physical. Any organic compound containing an amine, a hydroxyl, a hemiacetal, or a carboxylic acid moiety will react with either trifluoroacetic anhydride or methanolic HCl. Those derivatives with boiling points between approximately 100° and 200° and appropriate solubilities in trifluoropropylsilicone have the potential to interfere with the analysis, as do underivatized organic compounds with boiling points in that range. As shown in Table 4, a number of chemicals commonly used in the manipulation and purification of proteins do interfere with the analysis. Even laboratory dishwasher detergent can interfere. Zanetta, *et al.*, describe the peaks produced by SDS and fatty acids (39). In light of the number of substances which can produce peaks under the conditions of chromatography used here, it is surprising that the ketoses do not. Eklund, *et al.*, report that unmethanolysed fructose produces two small peaks in the pentose region using capillary columns. The response factor for

fructose was less than half that of the other sugars (77). There is some advantage in being able to identify the presence of fatty acids or certain buffer components in a sample, but in general this interference places considerable constraints on the choice of buffers for samples which are eventually to be analyzed for carbohydrate content. Precise measurement of peaks requires that they be significantly larger than the fluctuations in the baseline caused by tiny amounts of contaminants. The ability to remove such substances provides the ultimate lower limit to the amount of carbohydrate which can be analyzed.

In agreement with Tomana, *et al.*, (56), the present study shows that gas-liquid chromatographic analysis of N-acetylneuraminic acid is much less precise than analysis of the other sugars. The thiobarbituric acid assay is equally sensitive and more precise (41). The monosaccharide form of N-acetylneuraminic acid is required in this analysis. Acid hydrolysis was used to ensure complete release of the sugar because the immobilized neuraminidase from C. *perfringens* did not completely release the N-acetylneuraminic acid of human serum α_1-acid glycoprotein even though there was enough enzyme present to hydrolyze more than 300 times as much N-acetylneuraminic acid as was present. This neuraminidase is reported to be able to release neuraminic acids from all of the linkages found in human serum α_1-acid glycoprotein (36,55). The incomplete digestion seen here may be a result of steric hindrance by adjacent sugars (36) or an

alteration in the substrate specificity as a result of immobilization.

Neuraminic acids are partially degraded under the conditions of acid hydrolysis (53). The best standard to use under such conditions is one with a structure similar to the sample. NAN-lactose contains terminal N-acetylneuraminic acid in the same linkages found in glycoproteins (78). Even if the glycosidic linkage offers protection against degradation, the neuraminic acid of NAN-lactose should be cleaved and degraded at a rate similar to that of glycoprotein-linked neuraminic acid. The purity of the NAN-lactose was determined in the thiobarbituric acid assay after digestion with soluble neuraminidase from *C. perfringens*. Twenty percent less N-acetylneuraminic acid was recovered from NAN-lactose digested with acid than with neuraminidase, in contrast to the results with human serum α_1-acid glycoprotein, suggesting that the neuraminidase was cleaving the NAN-lactose completely.

Deglycosylation of Glycoproteins

Glycoproteins may be deglycosylated in liquid hydrogen fluoride (37) or in 70% hydrogen fluoride dissolved in pyridine (42). Use of the latter reagent eliminates the need for special reaction vessels and a closed system. The scaled-down reaction described in Chapter 2 is especially convenient because no precipitation of the protein is needed. Anhydrous hydrogen fluoride does not cleave proteins;

lysozyme and ribonuclease retain their activity after 2 hours of treatment (37). The technique has been used to study the effect of deglycosylation on luteotropin (LH), showing that partially deglycosylated LH retains the ability to bind to receptors but loses steroidogenic activity (79). Results showing that activity is lost should be interpreted with caution since the hydrogen fluoride may denature the protein (37).

Glycosidases can also be used to provide information about the structure and function of the carbohydrate portion of a glycoprotein (30,36,80,81). In the ideal system a sample of protein would be incubated with one or more purified glycosidases; at several time points aliquots would be taken for analysis of released sugars and measurement of biological and immunological activity. With this system, precise correlation of changes in activity and degree of deglycosylation would be possible.

The incubation mixture should be buffered to ensure that the pH will remain in a range tolerated by both the enzymes and the sample. Volatile buffers such as ammonium acetate cause the least interference with the carbohydrate analysis. Highly purified bovine serum albumin can be used as a protein carrier if needed. Gentamicin can be used as an antibiotic. Ammonium acetate is toxic to the bone marrow assay at a concentration of 5 mM but not at a concentration of 0.05 mM. However, purified epo has a specific activity of 70,000 units per mg so that the incubation mixture may

easily contain epo in sufficiently high concentration to permit the necessary dilution.

To be sure that the sugar released by the glycosidases actually came from the glycoprotein being studied, the enzyme preparation should contain significantly less carbohydrate than the sample. The highly purified marine tunicate enzymes met this criterion. The mixed glycosidase preparation from *S. pneumoniae*, however, contained large amounts of galactose, glucose, N-acetylglucosamine and N-acetylgalactosamine.

Inability of a glycosidase to remove sugar from a glycoprotein can provide structural evidence, but first it is necessary to confirm that the enzyme is active on the type of substrate suspected. Many glycosidases show much less activity on intact glycoproteins than on low molecular weight artificial substrates (36). The assay described in Chapter 2 can measure the activity of glycosidases on glycoprotein substrates with compositions similar to epo by separating free and bound sugars by micro-ultrafiltration and analyzing the carbohydrate content of both fractions. This assay is less rapid and less precise than the assays using radiolabelled substrate but does not require the synthesis of special substrates. The same procedure may be used for the carbohydrate analysis of the incubation mixtures.

Implications of the Carbohydrate Composition of Epo

Epo has a carbohydrate composition consistent with the presence of complex-type N-linked oligosaccharide chains. These chains normally consist of a core pentasaccharide of three mannose residues and two N-acetylglucosamine residues to which are attached a variable number of branches (78). The branches normally consist of chains containing, from the non-reducing terminus, N-acetylneuraminic acid, galactose, and N-acetylglucosamine. Fucose residues may be attached to N-acetylglucosamines at any point in the chain. Branches may be incomplete. Epo contains more fucose and N-acetylneuraminic acid per mole than is found in most serum glycoproteins (82). In α-epo there are more residues of N-acetylneuraminic acid than of galactose, which conflicts with the model of complex chains described above. However, linkage of N-acetylneuraminic acid to N-acetylglucosamine as well as galactose has been described (55). Possibly epo contains N-acetylneuraminic acid linked to both. In view of the absence of N-acetylgalactosamine, findings of O-glycosidically linked oligosaccaride in epo would be surprising. There is less N-acetylglucosamine than would have been expected from the amount of galactose and mannose present. Release of the N-glycosidically linked residues would not have occurred under the mild conditions of methanolysis used in this study.

Espada, *et al.*, have reported that the human urinary epo which they purified was 13.0% hexose, 8.9% hexosamine,

and 7.5% sialic acid (83). Their preparation has one-tenth the specific activity of the epo used in this study and was significantly different in amino acid composition. The difference in amino acid composition would suggest that the two preparations are fundamentally different. No evidence for heterogeneity has been found in the epo used in the present study.

Goldwasser reported finding 4% mannose, 6% galactose, 4% N-acetylglucosamine, and 10% sialic acid in a preparation of purified sheep plasma epo, but no fucose (84). With the exception of fucose, the values are very similar to those reported here for human urinary epo. Species differences in the external portions of the oligosaccharide chains including differences in fucose content been reported for human serum α_1-acid glycoprotein (55).

α-Epo contains significantly more N-acetylglucosamine and N-acetylneuraminic acid than β-epo. These differences could represent either the presence of an additional oligosaccharide chain in the α-epo or the presence of more highly branched chains. These differences do not account for the observation by Miyake, *et. al.*, that β-epo migrates more rapidly than α-epo on polyacrylamide gel electrophoresis at pH 9. If the difference in N-acetylneuraminic acid content were the only basis for the differing mobilities, α-epo would have a larger mobility than β-epo. Additional differences between the two forms must exist to account for the variation in mobilities.

Comparison of the Functions of Carbohydrate in Epo and Other Glycoproteins

While the function of the carbohydrate chains of most glycoproteins remains undetermined, three possible results of glycosylation have been well established. Glycosylation of other proteins alters the physical properties of the protein, such as tertiary structure or charge density. The biological behavior of some proteins remains unchanged whether or not carbohydrate chains are attached. Attachment of specific sugar sequences to some proteins labels them with extracellular routing signals. The problem of identifying these changes in a given glycoprotein may be approached either by removing the sugar from the intact protein or by examining polypeptides that lack the carbohydrate as a result of mutation or synthesis in the presence of compounds such as tunicamycin, an inhibitor of glycosylation of asparagine residues (85). In studying epo, we have taken the former approach because cell cultures in which epo synthesis can be manipulated are not currently available.

A number of proteins have the same biological activity whether or not they are fully glycosylated (27-29,86-92). Epo deglycosylated enzymatically retains 50% to 70% of its activity *in vitro*. Bovine pancreatic ribonuclease exists in four isozymes, one containing no sugar and the others containing different amounts of sugar, all in the same tryptic peptide. No difference in the activities of the isozymes has been discovered (86). Much of the carbohydrate has been

removed from soybean agglutinin (27), alkaline phosphatase (28), and human urinary colony-stimulating factor (29) without altering their biological activities. Human interferon treated with periodate (87) or glycosidases (88) retains its antiviral activity. Addition of tunicamycin to cell cultures does not prevent synthesis of active HLA antigens (89), human (90) or mouse (91) interferon, or a mouse L-cell protein stimulating differentiation of mouse myeloid leukemia cells (92).

Although deglycosylated epo retains much of its activity *in vitro*, the deglycosylated hormone is not active *in vivo*. Furthermore, the present experiments do not differentiate between a uniform decrease in the activity of all molecules and the total inactivation of a portion of the sample while the remainder retains full activity. Polyacrylamide gel electrophoresis of deglycosylated [^{125}I]iodoepo shows that no native epo remains. However, HPLC studies show that only 10% to 15% of the deglycosylated epo appears in peak B. If the only active material in the samples of deglycosylated epo is found in peak B, the deglycosylated epo has a specific activity 4 to 6 times that of native epo. The aggregate of native epo, peak A, is not active (57).

Some proteins must be glycosylated to assume their active form (30,31,79,80,93,94). In general, loss of the carbohydrate probably alters the conformation of these proteins. Studies of which sugars are involved in such

tertiary interactions can provide clues to methods of stabilizing polypeptides in a biologically active conformation. Georghegan, *et al.* provide an unusual explanation in the following series of experiments showing that intact carbohydrate chains of the antifreeze glycoproteins found in polar fish are needed for solutions of those proteins to exhibit anomalous freezing-point depressions (80). Removal of the chains by β-elimination, removal of the terminal galactose residues by β-galactosidase treatment, acetylation of hydroxyl groups, or introduction of a charged group onto C-6 of the galactose residues all destroy the antifreeze activity. Introduction of an uncharged aldehyde at C-6 of the galactose residues does not affect antifreeze activity. The authors suggest that the galactose hydroxyl groups coat nucleation sites and prevent ice crystal formation. Such a detailed explanation of carbohydrate involvement in biological behavior has been obtained for few proteins other than the antifreeze glycoproteins.

Sialic acids, which are negatively charged at physiological pH and have a large effect on the charge density of a protein, were predicted to affect protein conformation. The high neuraminic acid content of mucins was hypothesized to be partially responsible for their viscosity (49,95). However, more recent studies have found that mucins treated with neuraminidase have unaltered viscosity and elasticity despite their decreased molecular charge (96,97). In addition, removal of neuraminic acid has little effect on the *in*

vitro activity of epo (3), human urinary colony stimulating factor (29), human chorionic gonadotropin (hCG) (30), or human interferon (90). Apparently changes in charge density do not alter the conformation of these proteins in a way that would affect biological activity.

A deglycosylated protein may lose one of its activities and not another, suggesting that the loss is the result of localized changes in the protein. Although human leukocyte interferon grown in cells treated with tunicamycin has as much antiviral activity as that grown in untreated cells, the non-N-glycosylated interferon has lost its ability to bind polyribonucleotides and, judging from altered binding to phenylagarose, is less hydrophobic than glycosylated interferon (90). Rabbit IgG treated with N-acetylglucosamine amidohydrolase, which cleaves the N-glycosidic bond between N-acetylglucosamine and asparagine, retains normal antigen binding characteristics but is completely unable to bind Clq, the first component of complement (31). As a result, deglyco-IgG cannot initiate the classical pathway of the complement cascade or induce complement-mediated cytolysis.

The gonadotropins and thyrotropin consist of identical α subunits and hormone-specific β subunits, both of which are required for activity. Each hormone loses certain of its activities when large amounts of carbohydrate are removed. HCG treated with glycosidases loses its ablility to stimulate cAMP production or progesterone secretion after

removal of the penultimate residues but retains partial receptor binding even when only the mannose-N-acetylglucosamine cores remain (30). Likewise, ovine luteinizing hormone (LH) which has been partially deglycosylated in anhydrous hydrogen fluoride retains 40% to 50% of its ability to displace [^{125}I]-iodoLH from testicular membranes but loses its ability to stimulate testosterone synthesis (79). This deglyco-LH inhibits LH-stimulated ovulation *in vivo* (98). More complete deglycosylation of LH using trifluoromethanesulfonic acid abolishes the residual receptor binding (93). The unglycosylated α subunit formed when thyrotropin (TSH) is synthesized in the presence of tunicamycin is unable to combine with TSH β subunit (94). Recombination of partially deglycosylated LH subunits occurs normally (79).

Epo exhibits a variety of effects on erythroid precursor cells in a definite sequence. The most rapid response to epo is an increase in RNA synthesis. Iron uptake and heme synthesis, the characteristics measured by the *in vitro* bone marrow assay, increase much later. Since deglycosylation may affect one activity and not another, future experiments should examine the ability of deglycosylated epo to stimulate RNA and DNA synthesis. A modified epo which could uncouple the various effects of epo on its target cells would provide a powerful tool for studying the molecular mechanism of epo action.

Increased sensitivity to protease digestion is believed to reflect increased accessibility of the cleavage sites to

the enzyme. Asialo-epo is more trypsin-sensitive than native epo (3). IgM (33) and pro-opiocortin (34) synthesized by *in vitro* translation systems without post-translational processing are also more sensitive to trypsin digestion than their glycosylated analogs. Degradation may also be more random in non-glycosylated proteins. Digestion of glycosylated pro-opiocortin produces discrete species corresponding to the high-molecular weight ACTH forms, while the digested aglyco-pro-opiocortin contains no discrete species (34).

Changes in the stability of a protein toward denaturation and aggregation also relect conformational changes. Asialo-epo has a half-life at 100° approximately half that of native epo (3). In the current study, epo treated with the *S. pneumoniae* mixed glycosidases developed much larger quantities of the high molecular weight aggregate than epo incubated for the same period of time in the absence of enzymes. The epo was not merely binding to some component of the enzyme preparation; a sample chromatographed two minutes after mixing the epo and enzymes did not show any accumulation of aggregate.

Specific sugars serve as routing signals for glycoproteins. As a result, alteration of carbohydrate chains may change *in vivo* compartmentalization of a glycoprotein without affecting its intrinsic activity. The presence of mannose-6-phosphate in the oligosaccharides causes pinocytosis of glycoproteins into lysosomes (99). Terminal galactose

and fucose direct endocytosis of proteins by hepatocytes (15,17,35) while terminal mannose and N-acetylglucosamine direct endocytosis of proteins by hepatic sinusoidal cells, part of the reticuloendothelial system (16,35). Neuraminic acids prevent glycoproteins from binding to the receptors for other sugars (15). In a similar manner, terminal neuraminic acid residues prevent hepatic binding protein from binding its own galactose residues and inactivating itself (100). A previous study has shown that asialo-epo is rapidly removed from the bloodstream and is not active *in vivo* (3). Epo treated with *S. pneumoniae* glycosidases is also inactive *in vivo*, although it is partially active *in vitro*. Although it is not possible to measure directly the extent of deglycosylation of the enzyme-treated epo, the apparent molecular weight is 28,500, while that reported for asialo-epo is 34,000. The difference would represent removal of 85% of the internal sugars. The *S. pneumoniae* glycosidases are not capable of breaking N-glycosidic bonds. Any residue linked to asparagine would not be removed. Possibly the sugars which remain direct the clearance *in vivo* and prevent expression of the activity seen *in vitro*.

By understanding the mechanisms of carbohydrate-directed compartmentalization of glycoproteins, it may be possible to direct protein replacement therapy to the proper cells by attachment of appropriate carbohydrate chains. Doebber, *et al.*, have coupled chemically synthesized Man_3Lys_2 to β-glucocerebrosidase without affecting enzyme

activity (101). The derivatized enzyme is taken up by macrophages *in vitro* 4 times as fast as underivatized enzyme. *In vivo*, the derivatized enzyme is cleared rapidly from the bloodstream, apparently by the reticuloendothelial system. Marsh, *et al.*, have coupled lactose and NAN-lactose to L-asparaginase from *Escherichia coli* and and demonstrated that the lactose derivative is cleared from the bloodstream more quickly and the NAN-lactose derivative less quickly than the untreated enzyme (102). The results presented here suggest that deglycosylated epo may retain its ability to stimulate erythropoiesis but lack the stability to survive *in vivo*. If the presence of carbohydrate is altering the clearance of epo, it may be possible to modify non-glycosylated hormone with non-specifically located carbohydrate chains as described above in order to prolong its survival in the bloodstream.

Summary

Epo is a glycoprotein containing 25% to 30% carbohydrate with a composition typical of glycoproteins bearing the complex type N-linked oligosaccharide chains. However, epo contains more fucose and N-acetylneuraminic acid than is normally found in serum glycoproteins. Epo exists in two forms which are separated by chromatography on hydroxylapatite and have differing mobilities on polyacrylamide gel electrophoresis at pH 9 (2). These forms show significant differences in N-acetylglucosamine and N-acetylgalactosamine

content, but those differences do not explain the difference in mobility.

Epo deglycosylated using hydrogen fluoride dissolved in pyridine loses at least 75% of the carbohydrate and 99% of its original *in vitro* activity. However, SDS-polyacrylamide gel electrophoresis shows that there has been considerable degradation of the protein. Digestion of the hormone with *S. pneumoniae* mixed glycosidases reduces the apparent molecular weight from 39,000 to 28,500. The glycosidase-treated epo retains 50% to 70% of its activity *in vitro* but is inactive *in vivo*. The glycosidase-treated epo aggregates much more readily than native epo; the ratio of aggregate to monomer in the enzyme-treated samples is as much as 25 times that of an untreated sample. These results suggest that deglycosylated epo may retain its intrinsic ability to stimulate erythropoiesis but may lack the stability *in vivo* required for successful hormone replacement therapy.

TABLE 1

CHARACTERISTICS OF PEAKS PRODUCED BY GAS-LIQUID CHROMATOGRAPHY OF TRIMETHYLSILYL ETHERS OF STANDARD METHYL GLYCOSIDES

Identification of Peaks		Relative Retention Times ±Std. Dev.	Relative Proportion of Peaks
fucose	1	0.273 ±0.0033	0.15
	2	0.300 ±0.0031	0.51
	3	0.334 ±0.0035	0.34
mannose	1	0.667 ±0.0029	0.95
	2	0.716 ±0.0058	0.05
galactose	1	0.688 ±0.0026	0.23
	2	0.744 ±0.0041	0.58
	3	0.804 ±0.0034	0.19
glucose	1	0.844 ±0.0023	0.71
	2	0.891 ±0.0038	0.29
mannitol	1	1.000*	0.93
	2	1.086 ±0.0031	0.07
galactosamine	1	0.665	0.05
	2	1.237 ±0.0042	0.95
glucosamine	1	0.735 ±0.0087	0.33
	2	1.051 ±0.0014	0.12
	3	1.196	0.55
N-acetylgalactosamine	1	1.061 ±0.0024	0.13
	2	1.145 ±0.0017	0.87
N-acetylglucosamine	1	1.116 ±0.0049	0.07
	2	1.203 ±0.0010	0.93
N-acetylneuraminic acid	1	1.681 ±0.0112	0.66
	2	1.755 ±0.0144	0.34

*The absolute retention time of mannitol was 27.00 ±0.218 minutes.

TABLE 2

CHARACTERISTICS OF PEAKS PRODUCED BY GAS-LIQUID CHROMATOGRAPHY OF TRIFLUOROACETATE ESTERS OF STANDARD METHYL GLYCOSIDES

Identification of Peaks		Relative Retention Times ±Std. Dev.	Relative Proportion of Peaks
fucose	1	0.583 ±0.0030	0.72
	2	0.755 ±0.0031	0.28
inositol		1.000*	1.00
galactose	1	1.182 ±0.0047	0.13
	2	1.241 ±0.0040	0.63
	3	1.425 ±0.0043	0.25
mannose	1	1.275 ±0.0066	0.94
	2	1.466 ±0.0050	0.06
glucose	1	1.312 ±0.0040	0.68
	2	1.392 ±0.0037	0.32
glucosamine	1	1.224	0.08
	2	1.804	0.76
	3	2.148	0.16
galactosamine	1	1.397	0.13
	2	1.943	0.63
	3	1.970	0.24
N-acetylglucosamine	1	2.067 ±0.0080	0.97
	2	2.148 ±0.0063	0.03
N-acetylgalactosamine	1	1.914 ±0.0048	0.13
	2	1.971 ±0.0049	0.11
	3	2.022 ±0.0055	0.08
	4	2.141 ±0.0074	0.68
N-acetylneuraminic acid	1	2.552 ±0.0045	0.04
	2	2.889 ±0.0062	0.96

*The absolute retention time of inositol was 30.63 ±0.231 minutes.

TABLE 3

CHARACTERISTICS OF PEAKS PRODUCED BY GAS-LIQUID CHROMATOGRAPHY OF TRIFLUOROACETATE ESTERS OF SOME UNUSUAL METHYL GLYCOSIDES

Identification of Peaks	Relative Retention Times*	Relative Proportion of Peaks
rhamnose 1	0.490	0.90
2	0.644	0.10
2-deoxyglucose 1	1.042	0.87
2	1.082	0.13
glucoheptose 1	1.455	0.67
2	1.716	0.33
sedoheptulose	1.593	1.00
mannosamine 1	1.612	0.14
2	2.065	0.45
3	2.212	0.24
4	2.315	0.17
mannoheptulose	1.914	1.00

*The absolute retention time of inositol was 26.50 ±0.118 minutes.

TABLE 4

RELATIVE RETENTION TIMES OF SUBSTANCES INTERFERING WITH CARBOHYDRATE ANALYSIS

Identification of Peaks	Relative Retention Times
Tris-hydroxymethylaminomethane 1	0.430
2	1.109
Ethylenediaminetetraacetic acid 1	0.949
2	1.028
3	1.282
4	2.359
Citric acid 1	1.220
2	1.360
Polyethylene glycol 1	1.254
2	1.643
3	1.745
4	1.900
5	2.994
Trichloroacetic acid 1	1.619
2	1.582
3	2.150
4	2.217
Contaminants in 0.1 M HCl, reagent grade	1.700
	2.164
	2.223
Dishwashing detergent	0.981
	1.28
	1.65
	2.40

TABLE 5

RELATIVE RESPONSE FACTORS FOR TRIFLUOROACETATE ESTERS OF METHYL GLYCOSIDES

Parent Sugar	Mass Response Factor*	Molar Response Factor*
fucose	0.63 ±0.043	0.57 ±0.040
galactose	0.90 ±0.042	0.90 ±0.042
mannose	0.93 ±0.041	0.93 ±0.041
glucose	1.06 ±0.045	1.06 ±0.045
N-acetylglucosamine	0.72 ±0.090	0.88 ±0.111
N-acetylneuraminic acid	0.38 ±0.082	0.65 ±0.142

*Area per ng of sugar/Area per ng of inositol
*Area per nmole sugar/Area per nmole inositol

TABLE 6

THE CARBOHYDRATE COMPOSITION OF ERYTHROPOIETIN

	COMPOSITION BY WEIGHT[a] ng sugar/μg erythropoietin		
	α Erythropoietin	β Erythropoietin	P[b]
fucose	18 ± 3.5	16 ±0.4	NSD[c]
galactose	56 ± 6.4	48 ±0.7	NSD
mannose	41 ± 6.0	34 ±4.2	NSD
N-acetylglucosamine	69 ± 0.9	48 ±3.2	P<.001
N-acetylneuraminic acid	130 ±17.7	95 ±7.6	P<.05
total sugar	313 ±25.8	241 ±4.9	P<.01
	MOLAR COMPOSITION moles sugar/mole erythropoietin		
fucose	4 ±0.8	4 ±0.1	
galactose	11 ±1.4	11 ±0.1	
mannose	9 ±1.2	8 ±1.0	
N-acetylglucosamine	12 ±0.2	9 ±0.6	
N-acetylneuraminic acid	16 ±2.2	12 ±1.0	

[a]Using residue mass rather than mass of released sugar
[b]Probability that the samples of α and β erythropoietin have the same composition.
[c]No significant difference

TABLE 7

THE N-ACETYLNEURAMINIC ACID CONTENT

OF ERYTHROPOIETIN

	Moles Sugar/Mole Erythropoietin	
	By Thiobarbituric Acid Assay	By Gas-Liquid Chromatography
α-erythropoietin	15 ±1.1	16 ±2.2
β-erythropoietin	13 ±0.5	12 ±1.0
P*	P<.05	P<.05

*Probability that the samples of α and β erythropoietin have the same composition

TABLE 8

REMOVAL OF SUGAR FROM ERYTHROPOIETIN BY HYDROGEN FLUORIDE DEGLYCOSYLATION

	Composition of β-erythropoietin ng sugar per µg protein	Sugar Remaining After Treatment ng sugar per µg protein	Percent of Sugar Removed
fucose	16	0	100
galactose	48	11	77
mannose	34	6	81
N-acetylglucosamine	69	3	93
N-acetylneuraminic acid	95	41	58
total sugar	240	61	75

TABLE 9

CHARACTERISTICS OF PEAKS PRODUCED BY GAS-LIQUID CHROMATOGRAPHY OF TRIFLUOROACETATE ESTERS OF MONOSACCHARIDES

Identification of Peaks	Relative Retention Times[a]	Relative Proportion of Peaks
fucose 1	0.595	0.44
2	0.742	0.33
3	0.847	0.23
galactose 1	1.266	0.29
2	1.464	0.50
3	1.525	0.11
4	1.656	0.11
glucose 1	1.280	0.48
2	1.436	0.52
mannose 1	1.338	0.65
2	1.613	0.35
N-acetylglucosamine[b]	1.913	1.00
N-acetylgalactosamine[c]	2.046	1.00

[a]The absolute retention time of inositol was 27.57 ±0.085 minutes.
[b]Glucosamine has the same retention time.
[c]Galactosamine has the same retention time.

TABLE 10

SEPARATION OF FREE SUGARS FROM PROTEIN BY MICRO-ULTRAFILTRATION

	μg SUGAR RECOVERED					
	Monosaccharides[a]		Bovine Serum Albumin		Intact Ovalbumin	
	Filtrate	Retentate	Filtrate	Retentate	Filtrate	Retentate
fucose	1.6	---	0.1	---	---	---
galactose	1.0	trace	---	trace	---	3.0
mannose	1.0	trace	0.1	---	---	5.7
glucose	1.5	0.2[b]	0.3	0.3[b]	---	0.4
N-acetylglucosamine	1.0	---	---	0.4[b]	---	2.9
N-acetylgalactosamine	1.6	0.4[b]	---	0.7[b]	---	2.6
N-acetylneuraminic acid	0.8	---	---	---	---	---

[a]The original sample contained 1.0 μg of each sugar.
[b]Minimum amount of sugar in peak.

TABLE 11

APPARENT MOLECULAR WEIGHTS OF PEAKS PRODUCED BY MOLECULAR EXCLUSION CHROMATOGRAPHY OF [^{125}I]IODOERYTHROPOIETIN INCUBATED IN THE PRESENCE AND ABSENCE OF MIXED GLYCOSIDASES

Incubation Conditions	[^{125}I]iodo-erythropoietin Only		[^{125}I]iodo-erythropoietin Plus Glycosidases		
	Peak B	Peak C	Peak B	Peak C	Peak D
2 minutes, 20°	53,000	7,300	35,000	7,300	-----
15 minutes, 37°	48,000	6,000	23,000	6,000	-----
6 hours, 37°	43,000	7,300	29,000	7,300	3,500
21 hours, 37°	39,000	7,300	23,000	7,300	3,500

TABLE 12

THE BIOLOGICAL ACTIVITY OF

DEGLYCOSYLATED ERYTHROPOIETIN

Preparation	*In Vitro* Bone Marrow Assay, % of Control*	Radioimmuno-assay, % of Control*
A	60	ND*
B	65	49
C	71	43
D	48	31
Mean	61	41
±Std. Dev.	±9.8	±9.2

*Activity of erythropoietin incubated with mixed glycosidases as a percent of activity of erythropoietin incubated in the absence of glycosidases.
*Not measured.

FIGURE 1: Chromatogram from the Carbohydrate Analysis of a Mixture of Standard Monosaccharides. The mixture contained fucose (3.81 nmoles), galactose (6.94 nmoles), mannose, (6.94 nmoles), glucose (3.47 nmoles), N-acetylglucosamine (11.3 nmoles), N-acetylgalactosamine (2.83 nmoles), and N-acetylneuraminic acid (8.08 nmoles). Inositol (2.775 nmoles) was added as an internal standard. The number printed above each peak is the absolute retention time in minutes.

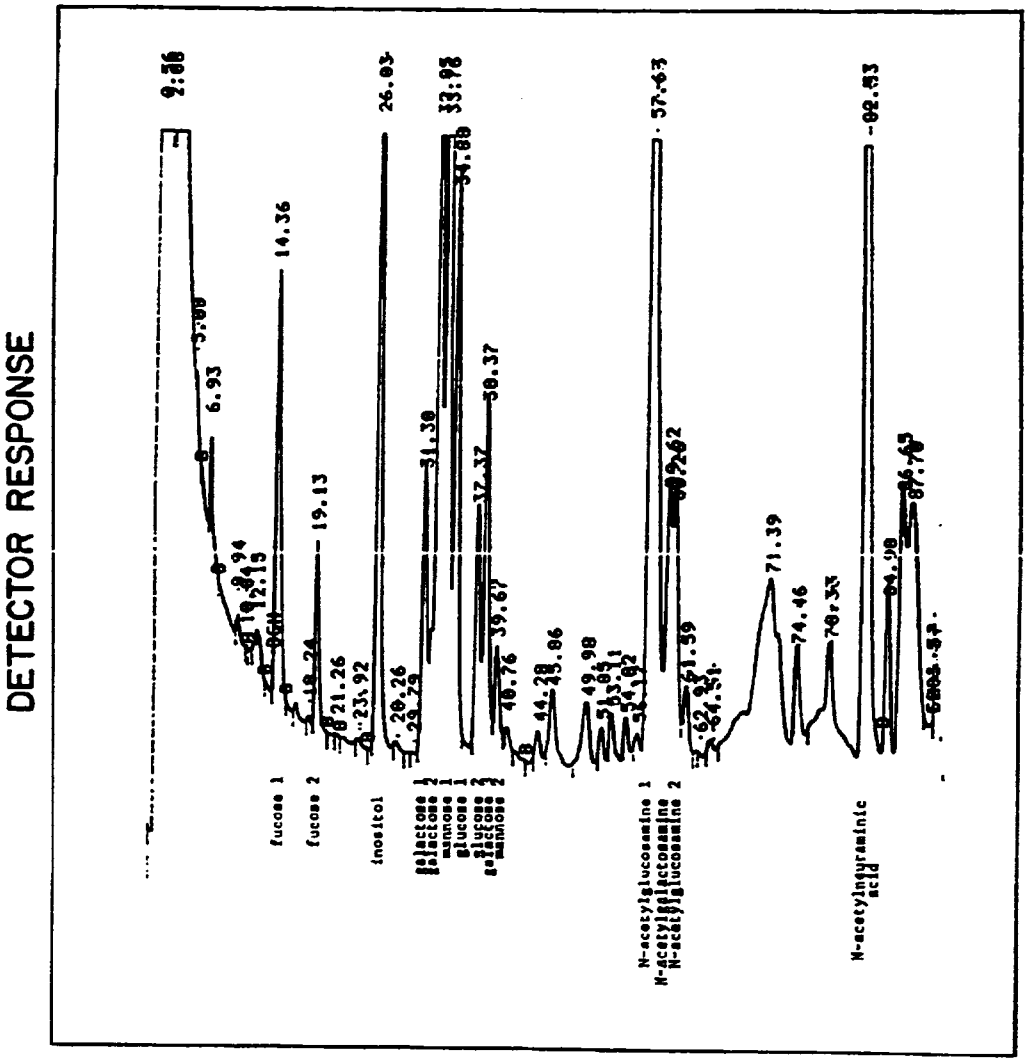

DETECTOR RESPONSE
TIME, MINUTES
fucose 1
fucose 2
inositol
galactose 1
galactose 2
mannose 1
glucose 1
glucose 2
galactose 2
mannose 2
N-acetylglucosamine 1
N-acetylgalactosamine
N-acetylglucosamine 2
N-acetylneuraminic acid

FIGURE 2: Chromatogram from the Carbohydrate Analysis of β-Erythropoietin. A 0.435 nmole sample of β-erythropoietin was methanolysed and the released sugars analysed by gas-liquid chromatography. The arrows mark the position of the major peak of N-acetylgalactosamine and the minor peak of N-acetylglucosamine. Inositol (2.775 nmoles) was added as an internal standard. The number printed above each peak is the absolute retention time in minutes.

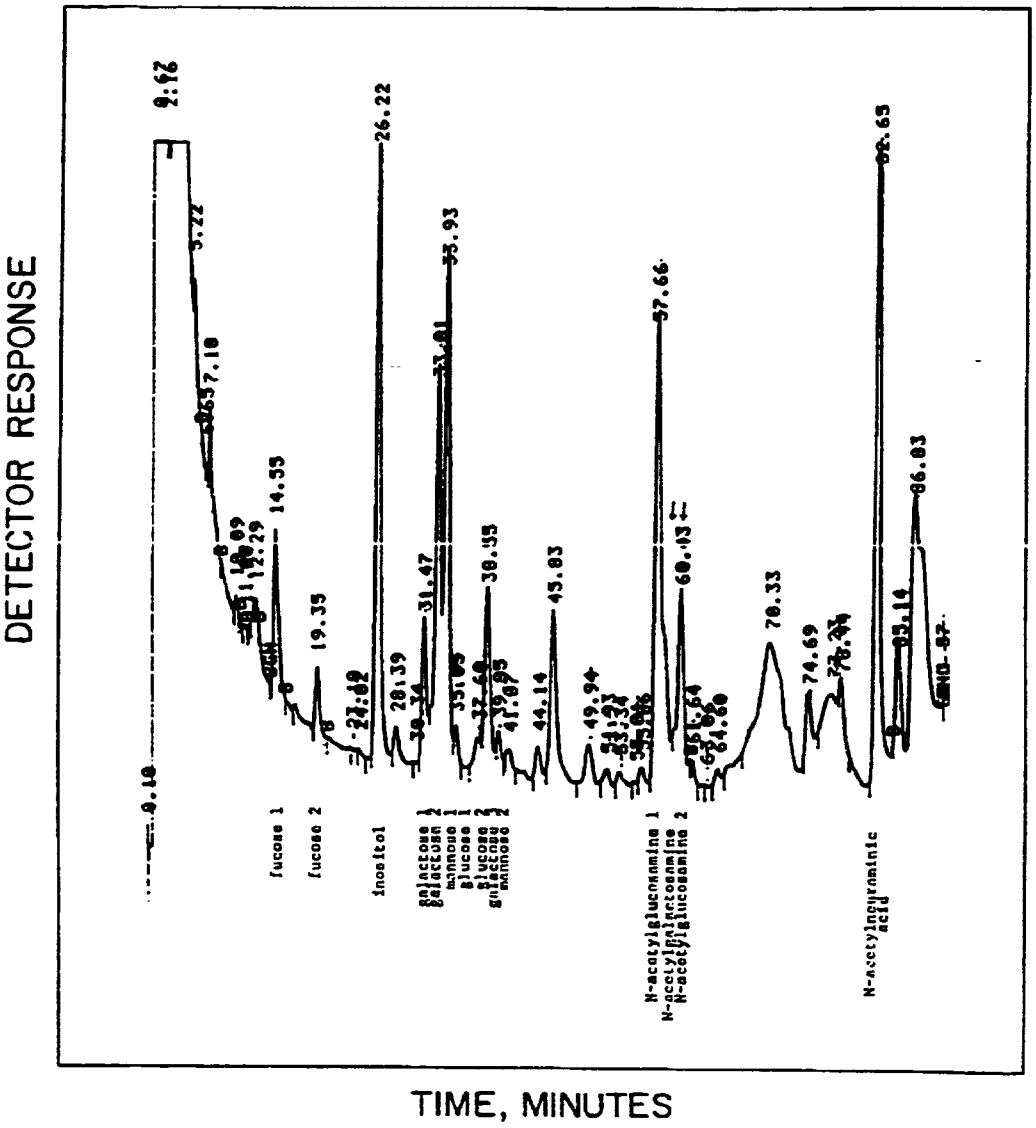

DETECTOR RESPONSE
TIME, MINUTES
fucose 1
fucose 2
inositol
N-acetylneuraminic acid

FIGURE 3: Chromatogram from the Carbohydrate Analysis of Deglycoslyated β-Erythropoietin. A 0.118 nmole sample of β-erythropoietin was treated with 70% anhydrous hydrogen fluoride in pyridine for 6 hours. Inositol (2.775 nmoles) was added as an internal standard. The number printed above each peak is the absolute retention time in minutes.

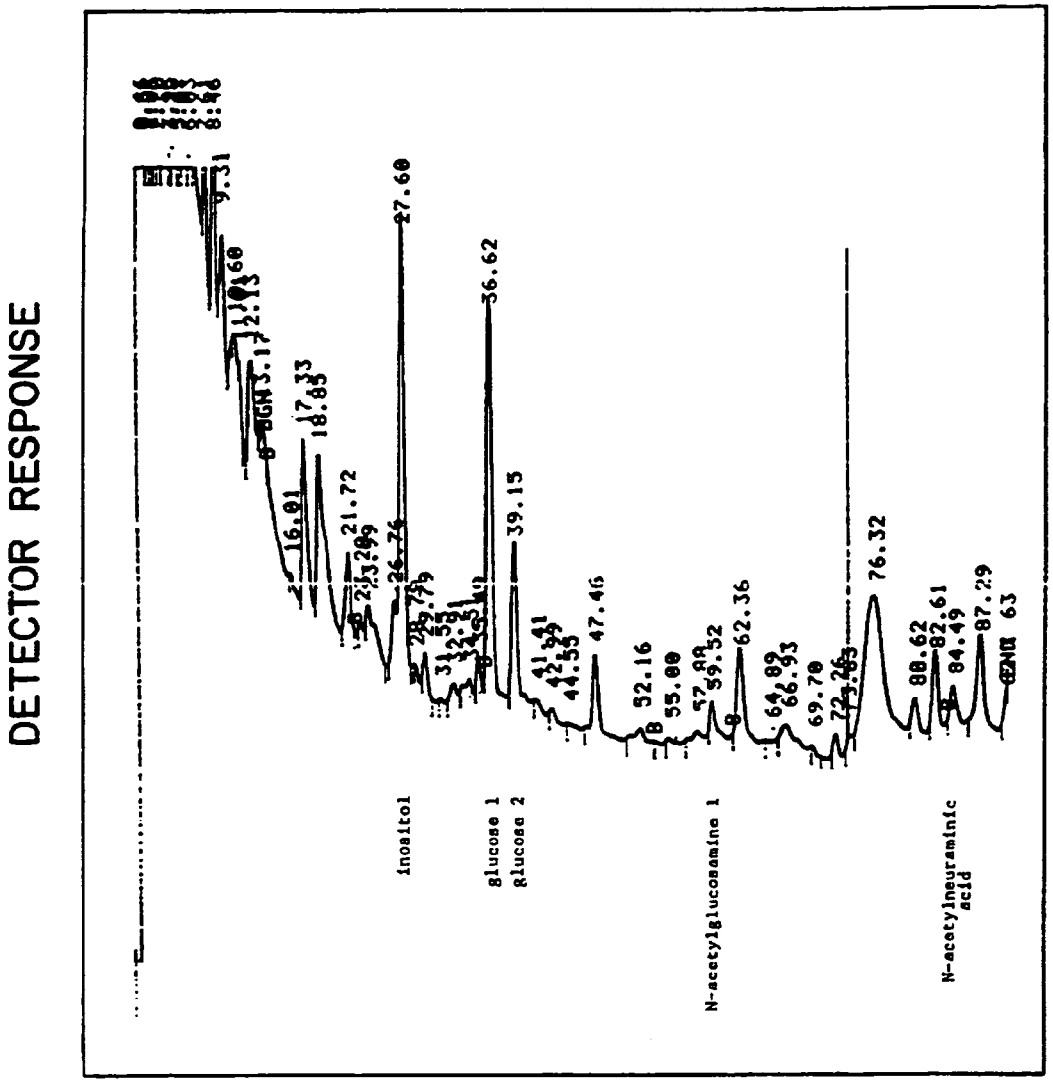

DETECTOR RESPONSE
TIME, MINUTES
9.31
16.01
17.33
18.85
21.72
26.76
27.60
36.62
39.15
47.46
52.16
55.00
59.52
62.36
76.32
80.62
82.61
84.49
87.29
inositol
glucose 1
glucose 2
N-acetylglucosamine 1
N-acetylneuraminic acid

FIGURE 4: Autoradiograph of Untreated and Deglycosylated [^{125}I]Iodoerythropoietin Electrophoresed in a 15% SDS-Polyacrylamide Gel. Lanes 1 and 11 contain ^{14}C-methylated lysozyme, chymotrypsinogen A, ovalbumin, bovine serum albumin, phosphorylase B, and myosin. Lanes 4 and 9 contain untreated [^{125}I]iodoerythropoietin. Lane 3 contains [^{125}I]iodoerythropoietin treated with 70% anhydrous hydrogen fluoride in pyridine for 6 hours. Lanes 5 to 7 contain [^{125}I]iodoerythropoietin incubated with *S. pneumoniae* glycosidases for 2, 15, and 360 minutes, respectively. Lane 8 contains [^{125}I]iodoerythropoietin incubated for 360 minutes in the absence of glycosidases.

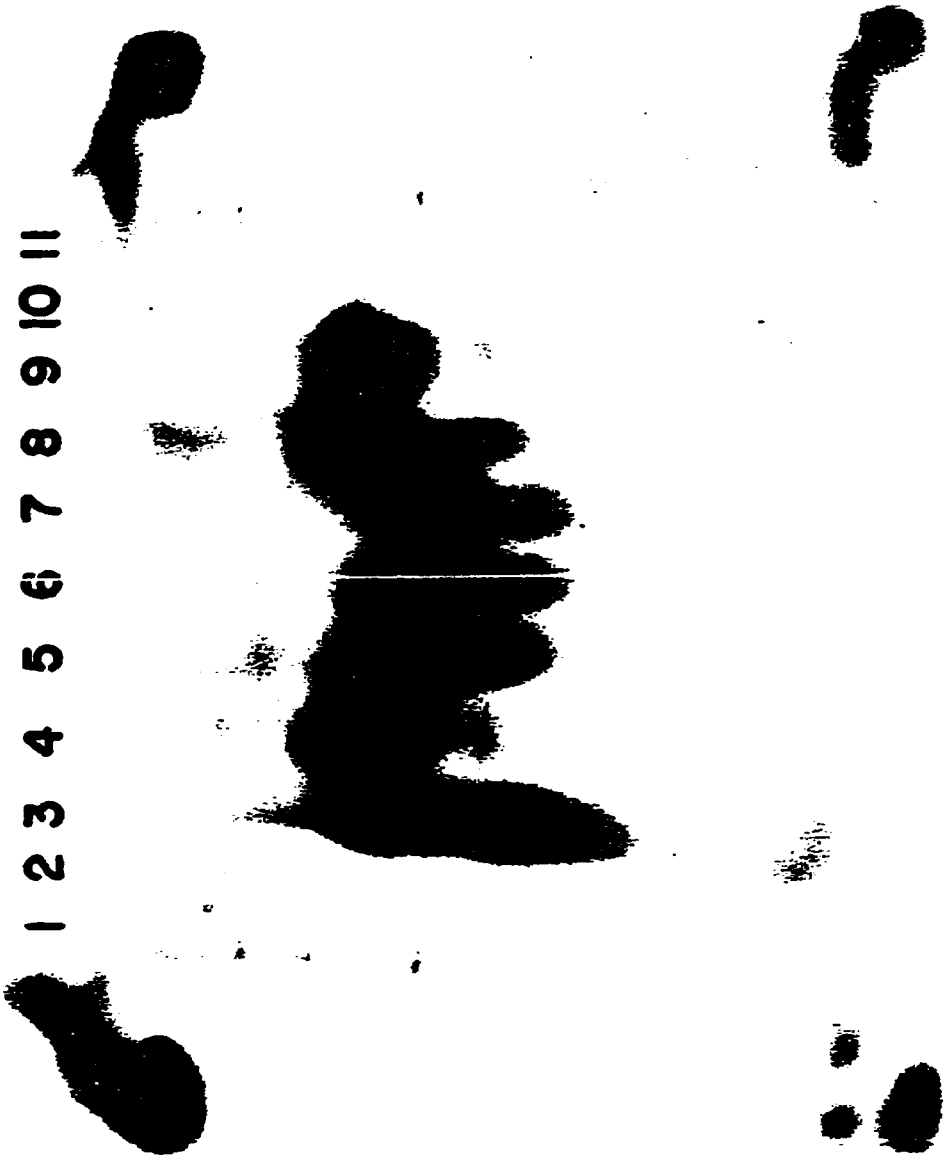

1 2 3 4 5 6 7 8 9 10 11

FIGURE 5: Profile of Untreated and Glycosidase-Treated [^{125}I]Iodoerythropoietin in a Sliced 15% SDS-Polyacrylamide Gel. Samples were electrophoresed in a 15% SDS-polyacrylamide gel along with a mixture of lysozyme, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin, and phosphorylase B. Gels were stained with Coomassie blue, destained, sliced, and counted. Those slices containing stained standards were recorded.
(- - - -)[^{125}I]iodoerythropoietin was incubated in 0.05 M cacodylate buffer, pH 6.5 for 14 hours at 37°.
(————)[^{125}I]iodoerythropoietin incubated with *S. pneumoniae* mixed glycosidases in 0.05 M cacodylate buffer, pH 6.5, for 14 hours at 37°.

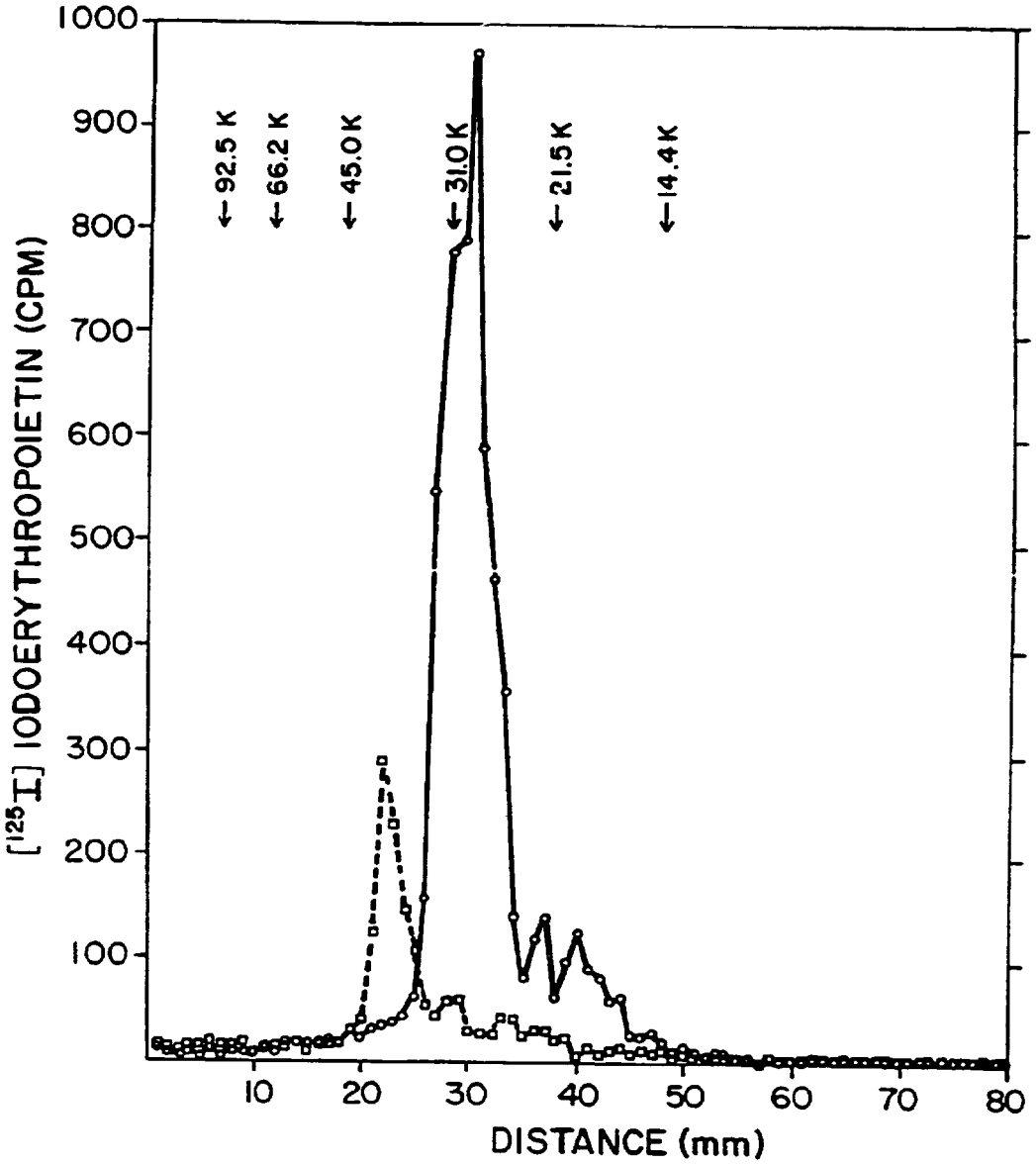

1000
900
800
700
600
500
400
300
200
100
[125I] IODOERYTHROPOIETIN (CPM)
← 92.5 K
← 66.2 K
← 45.0 K
← 31.0 K
← 21.5 K
← 14.4 K
10
20
30
40
50
60
70
80
DISTANCE (mm)

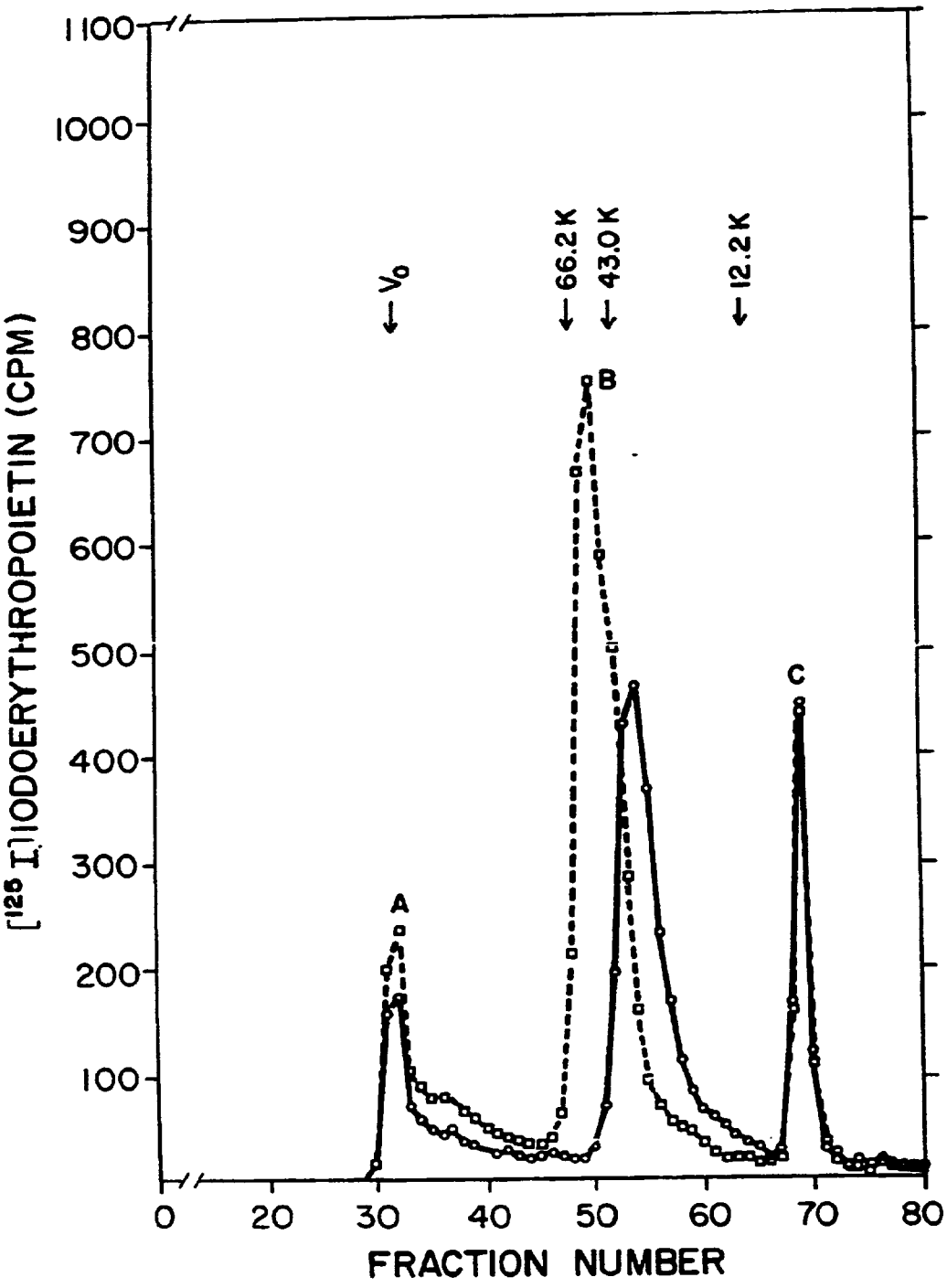
FIGURE 6: Molecular exclusion chromatography of [^{125}I]iodoerythropoietin incubated in the presence and absence of mixed glycosidases from *S. pneumoniae*.

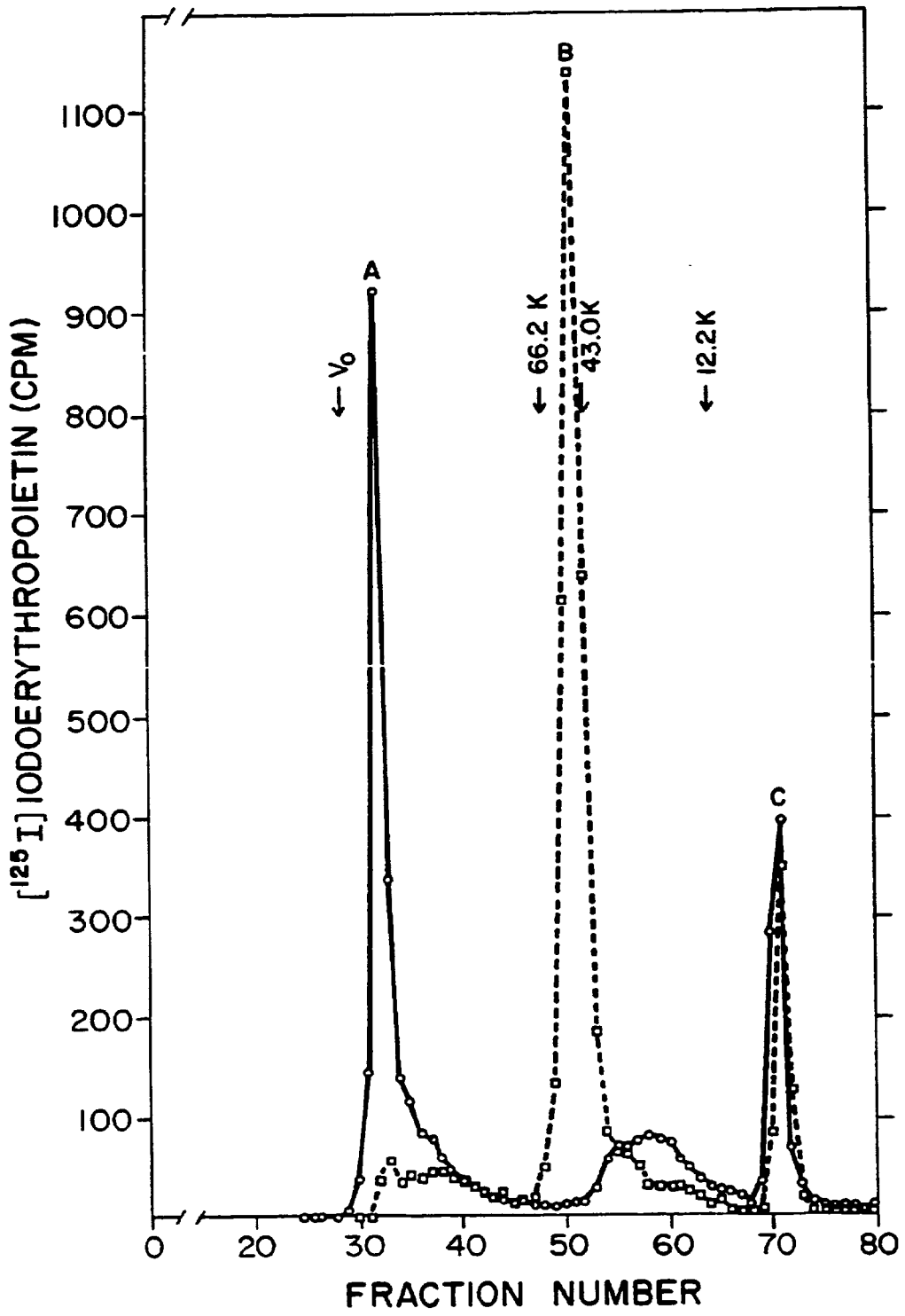
A. Samples mixed with 0.05 M cacodylate buffer, pH 6.5, or glycosidases and injected onto the chromatograph within 2 minutes. (- - - -) Erythropoietin only. (————) Erythropoietin mixed with glycosidases.

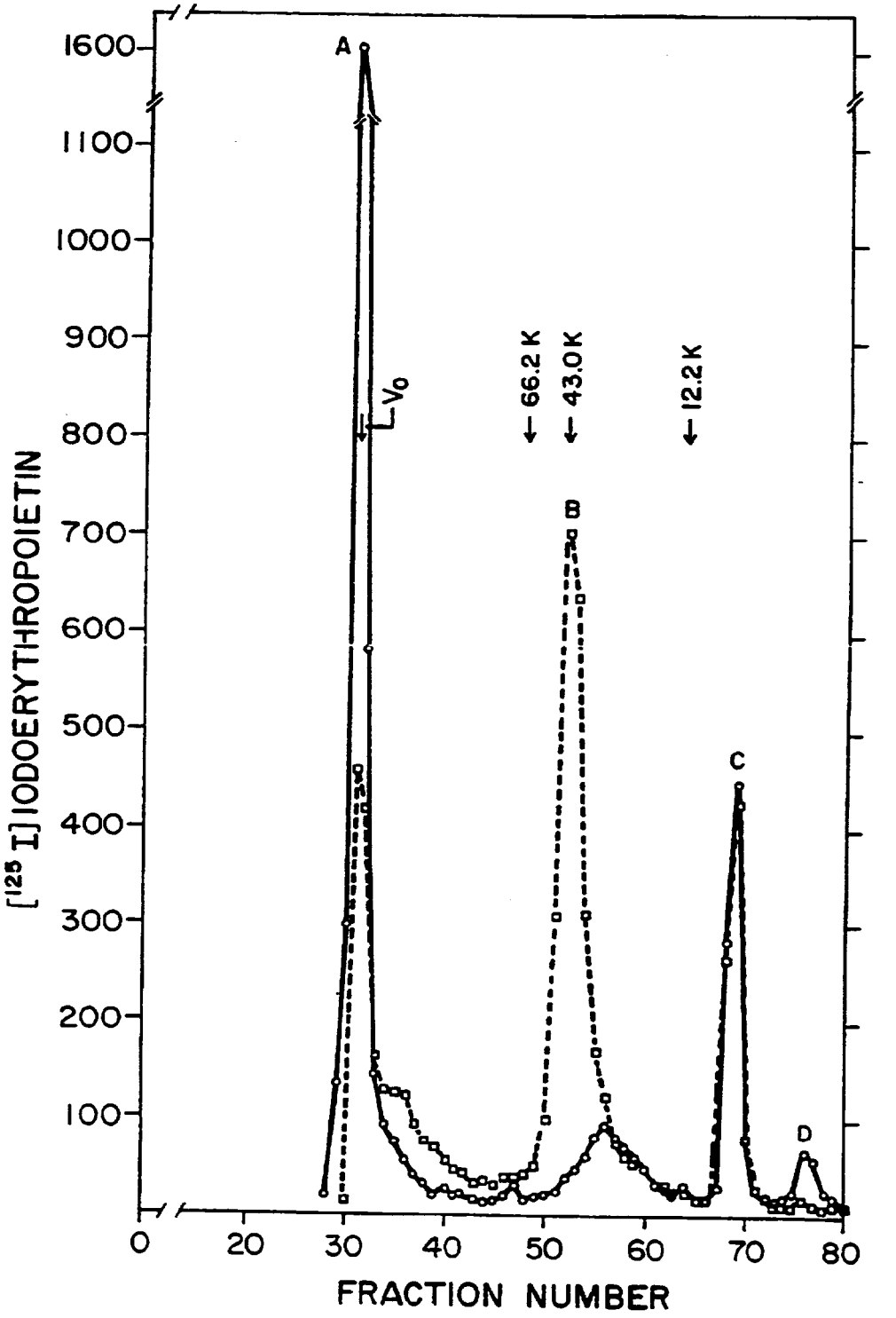
B. Samples incubated in 0.05 M cacodylate buffer, pH 6.5, for 15 minutes at 37°. (- - - -) Erythropoietin only. (————) Erythropoietin mixed with glycosidases.

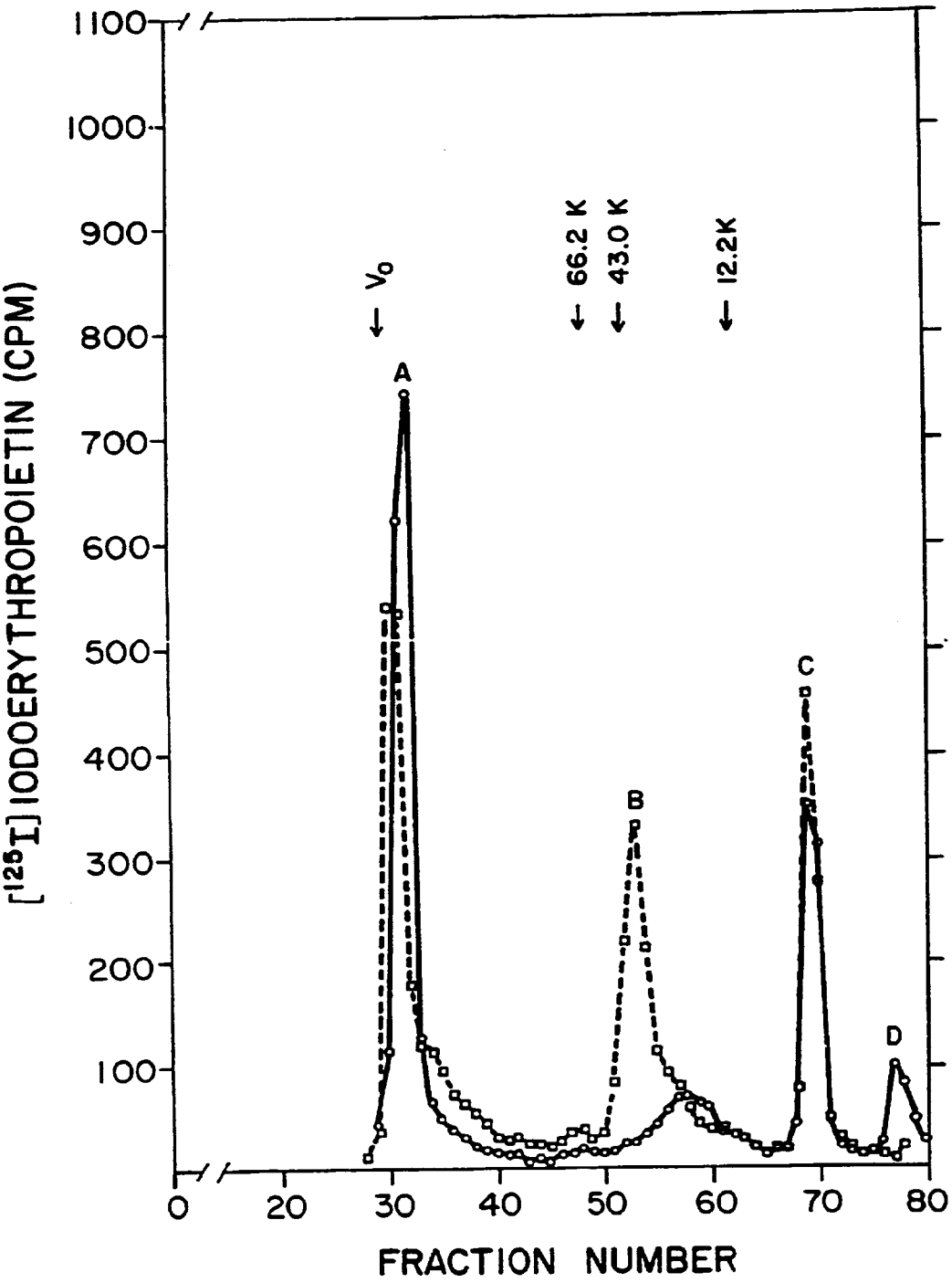
C. Samples incubated in 0.05 M cacodylate buffer, pH 6.5, for 6 hours at 37°. (- - - -) Erythropoietin only. (————) Erythropoietin mixed with glycosidases.

D. Samples incubated in 0.05 M cacodylate buffer, pH 6.5, for 21 hours at 37°. (- - - -) Erythropoietin only. (————) Erythropoietin mixed with glycosidases.


[125 I]IODOERYTHROPOIETIN (CPM)
1100
1000
900
800
700
600
500
400
300
200
100
0
20
30
40
50
60
70
80
FRACTION NUMBER
← V0
← 66.2 K
← 43.0 K
← 12.2 K
A
B
C


[125I] IODOERYTHROPOIETIN (CPM)
1100
1000
900
800
700
600
500
400
300
200
100
0
20
30
40
50
60
70
80
FRACTION NUMBER
A
B
C
← V0
← 66.2 K
43.0K
← 12.2K


[125 I]IODOERYTHROPOIETIN
1600
1100
1000
900
800
700
600
500
400
300
200
100
0
20
30
40
50
60
70
80
FRACTION NUMBER
A
B
C
D
Vo
66.2 K
43.0 K
12.2 K


1100
1000
900
800
700
600
500
400
300
200
100
0
20
30
40
50
60
70
80
[125I] IODOERYTHROPOIETIN (CPM)
FRACTION NUMBER
Vo
66.2 K
43.0 K
12.2K
A
B
C
D

APPENDIX

The molar response factor is defined as

$$RF_{x,i,m} = \frac{\Sigma A_x/m_x}{A_i/m_i}$$

and the mass response factor as

$$RF_{x,i,n} = \frac{\Sigma A_x/n_x}{A_i/n_i}$$

where x = sugar whose response is being measured

i = inositol

A = area of a peak associated with the sugar

m = moles of sugar in the sample

n = mass of sugar in the sample

These response factors may be used to quantitate the sugars in a glycoprotein as follows:

$$C_{x,i,m} = \frac{\Sigma A_x}{A_i} \frac{1}{RF_{x,i,m}} \frac{m_i}{m_p}$$

and

$$C_{x,i,n} = \frac{\Sigma A_x}{A_i} \frac{1}{RF_{x,i,n}} \frac{MW_x - 18.02}{MW_x} \frac{n_i}{n_p}$$

where x = sugar being measured

i = inositol

p = protein

A = area of a peak associated with the sugar

m = moles of substance in the sample

n = mass of substance in the sample

MW. = molecular weight of the sugar

The correction factor MW. - 18.02 is needed in calculating composition by weight to account for the fact that the sugars in a glycoprotein are in glycosidic linkage and have lost one molecule of water each.

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