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(72) Inventeurs/Inventors:
 DESNICK, ROBERT J., US;
 BISHOP, DAVID F., US;
 IOANNOU, YIANNIS A., US

(73) Propriétaire/Owner:
 THE MOUNT SINAI SCHOOL OF MEDICINE OF THE
 CITY UNIVERSITY OF NEW YORK, US

(74) Agent: OSLER, HOSKIN & HARCOURT LLP

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(57) **Abrégé/Abstract:**

The present invention involves the production of large quantities of human alpha-Gal A by cloning and expressing the alpha-Gal A coding sequence in eukaryotic host cell expression systems. The eukaryotic expression systems, and in particular the mammalian host cell expression system described herein provide for the appropriate cotranslational and posttranslational modifications required for proper processing, e.g., glycosylation, phosphorylation, etc. and sorting of the expression product so that an active enzyme is produced. In addition, the expression of fusion proteins which simplify purification is described. Using the methods described herein, the recombinant alpha-Gal A is secreted by the engineered host cells so that it is recovered from the culture medium in good yield. The alpha-Gal A produced in accordance with the invention may be used, but is not limited to, in the treatment in Fabry Disease; for the hydrolysis of alpha-galactosyl residues in glycoconjugates; and/or for the conversion of the blood group B antigen on erythrocytes to the blood group O antigen.



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<p>(21) International Application Number: PCT/US93/11539 (22) International Filing Date: 30 November 1993 (30.11.93) (30) Priority Data: 983,451 30 November 1992 (30.11.92) US (71) Applicant: THE MOUNT SINAI SCHOOL OF MEDICINE OF THE CITY UNIVERSITY OF NEW YORK [US/US]; One Gustave Levy Place, New York, NY 10029 (US). (72) Inventors: DESNICK, Robert, J.; 329 West 4th Street, New York, NY 10014 (US). BISHOP, David, F.; 306 East 96th Street, Apartment 17-E, New York, NY 10128 (US). IOANNOU, Yiannis, A.; 306 East 96th Street, Apartment 9-I, New York, NY 10128 (US). (74) Agents: CORUZZI, Laura, A. et al.; Pennie & Edmonds, 1155 Avenue of the Americas, New York, NY 10036 (US).</p>	<p>(81) Designated States: AU, BB, BG, BR, BY, CA, CZ, FI, HU, JP, KR, KZ, LK, LV, MG, MN, MW, NO, NZ, PL, RO, RU, SD, SK, UA, UZ, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report.</i></p>	
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CLONING AND EXPRESSION OF
BIOLOGICALLY ACTIVE ALPHA-GALACTOSIDASE A

1. INTRODUCTION

The present invention relates to the production of biologically active human α -Galactosidase (α -Gal A) involving cloning and expression of the genetic coding sequence for α -Gal A in eukaryotic expression systems which provide for proper post-translational modifications and processing of the expression product.

The invention is demonstrated herein by working examples in which high levels of α -Gal A were produced in mammalian expression systems. The α -Gal enzyme produced in accordance with the invention may be used for a variety of purposes, including but not limited to enzyme replacement therapy for Fabry Disease, industrial processes involving the hydrolysis of α -D--galactosyl residues of glycoconjugates, and for the conversion of the blood group B antigen on erythrocytes to the blood group O antigen.

2. BACKGROUND OF THE INVENTION

In the early 1970's, several investigators demonstrated the existence of two α -Galactosidase isozymes designated A and B, which hydrolyzed the α -galactosidic linkages in 4-MU-and/or ρ -NP- α -D--galactopyranosides (Kint, 1971, Arch. Int. Physiol. Biochem. 79: 633-644; Beutler & Kuhl, 1972, Amer. J. Hum. Genet. 24: 237-249; Romeo, et al., 1972, FEBS Lett. 27: 161-166; Wood & Nadler, 1972, Am. J. Hum. Genet. 24: 250-255; Ho, et al., 1972, Am. J. Hum. Genet. 24: 256-266; Desnick, et al., 1973, J. Lab. Clin. Med. 81: 157-171; and Desnick, et al., 1989, in The Metabolic Basis of Inherited Disease, Scriver, C.R., Beaudet, A.L. Sly, W.S. and Valle, D., eds, pp. 1751-1796, McGraw Hill, New York). In tissues, about 80%-90% of total α -Galactosidase (α -Gal) activity was

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due to a thermolabile, myoinositol-inhibitable α -Gal A isozyme, while a relatively thermostable, α -Gal B, accounted for the remainder. The two "isozymes" were separable by electrophoresis, isoelectric focusing, and ion exchange chromatography. After neuraminidase treatment, the electrophoretic migrations and pI value of α -Gal A and B were similar (Kint, 1971; Arch. Int. Physiol. Biochem. 79: 633-644), initially suggesting that the two enzymes were the differentially glycosylated products of the same gene. The finding that the purified glycoprotein enzymes had similar physical properties including subunit molecular weight (~46 kDa), homodimeric structures, and amino acid compositions also indicated their structural relatedness (Beutler & Kuhl, 1972, J. Biol. Chem. 247: 7195-7200; Callahan, et al., 1973, Biochem. Med. 7: 424-431; Dean, et al., 1977, Biochem. Biophys. Res. Comm. 77: 1411-1417; Schram, et al., 1977, Biochim. Biophys. Acta. 482: 138-144; Kusiak, et al., 1978, J. Biol. Chem. 253: 184-190; Dean, et al., 1979, J. Biol. Chem. 254: 10001-10005; and Bishop, et al., 1980, in Enzyme Therapy in Genetic Disease: 2, Desnick, R.J., ed., pp. 17-32, Alan R. Liss, Inc., New York).

However, the subsequent demonstration that polyclonal antibodies against α -Gal A or B did not cross-react with the other enzyme (Beutler & Kuhl, 1972, J. Biol. Chem. 247: 7195-7200; and Schram, et al., 1977, Biochim. Biophys. Acta. 482: 138-144); that only α -Gal A activity was deficient in hemizygotes with Fabry disease (Kint, 1971; Arch. Int. Physiol. Biochem. 79: 633-644; Beutler & Kuhl, 1972, Amer. J. Hum. Genet. 24: 237-249; Romeo, et al., 1972, FEBS Lett. 27: 161-166; Wood & Nadler, 1972, Am. J. Hum. Genet. 24: 250-255; Ho, et al., 1972, Am. J. Hum. Genet. 24: 256-266; Desnick, et al., 1973, J. Lab. Clin. Med. 81: 157-171;

Desnick, et al., 1989, in The Metabolic Basis of Inherited Disease, Scriver, C.R., Beaudet, A.L. Sly, W.S. and Valle, D., eds, pp. 1751-1796, McGraw Hill, New York; and, Beutler & Kuhl, 1972, J. Biol. Chem. 247: 7195-7200); and that the genes for α -Gal A and B mapped to different chromosomes (Desnick, et al., 1989, in The Metabolic Basis of Inherited Disease, Scriver, C.R., Beaudet, A.L. Sly, W.S. and Valle, D., eds, pp. 1751-1796, McGraw Hill, New York; deGroot, et al., 1978, Hum. Genet. 44: 305-312), clearly demonstrated that these enzymes were genetically distinct.

2.1. α -GAL A AND FABRY DISEASE

In Fabry disease, a lysosomal storage disease resulting from the deficient activity of α -Gal A, identification of the enzymatic defect in 1967 (Brady, et al., 1967, N. Eng. J. Med. 276: 1163) led to the first in vitro (Dawson, et al., 1973, Pediat. Res. 7: 694-690m) and in vivo (Mapes, et al., 1970, Science 169: 987) therapeutic trials of α -Gal A replacement in 1969 and 1970, respectively. These and subsequent trials (Mapes, et al., 1970, Science 169: 987; Desnick, et al., 1979, Proc. Natl. Acad. Sci. USA 76: 5326; and, Brady, et al., 1973, N. Engl. J. Med. 289: 9) demonstrated the biochemical effectiveness of direct enzyme replacement for this disease. Repeated injections of purified splenic and plasma α -Gal A (100,000 U/injection) were administered to affected hemizygotes over a four month period (Desnick, et al., 1979, Proc. Natl. Acad. Sci. USA 76: 5326). The results of these studies demonstrated that (a) the plasma clearance of the splenic form was 7 times faster than that of the plasma form (10 min vs 70 min); (b) compared to the splenic form of the enzyme,

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the plasma form effected a 25-fold greater depletion of plasma substrate over a markedly longer period (48 hours vs 1 hour); (c) there was no evidence of an immunologic response to six doses of either form, administered intravenously over a four month period to two affected hemizygotes; and (d) suggestive evidence was obtained indicating that stored tissue substrate was mobilized into the circulation following depletion by the plasma form, but not by the splenic form of the enzyme. Thus, the administered enzyme not only depleted the substrate from the circulation (a major site of accumulation), but also possibly mobilized the previously stored substrate from other depots into the circulation for subsequent clearance. These studies indicated the potential for eliminating, or significantly reducing, the pathological glycolipid storage by repeated enzyme replacement.

However, the biochemical and clinical effectiveness of enzyme replacement in Fabry disease has not been demonstrated due to the lack of sufficient human enzyme for adequate doses and long-term evaluation.

2.2. THE α -Gal A ENZYME

The α -Gal A human enzyme has a molecular weight of approximately 101,000 Da. On SDS gel electrophoresis it migrates as a single band of approximately 49,000 Da indicating the enzyme is a homodimer (Bishop & Desnick, 1981, J. Biol. Chem. 256: 1307). α -Gal A is synthesized as a 50,500 Da precursor containing phosphorylated endoglycosidase H sensitive oligosaccharides. This precursor is processed to a mature form of about 46,000 Da within 3-7 days after its synthesis. The intermediates of this processing have not been defined (Lemansky, et

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al., 1987, J. Biol. Chem. 262: 2062). As with many lysosomal enzymes, α -Gal A is targeted to the lysosome via the mannose-6-phosphate receptor. This is
5 evidenced by the high secretion rate of this enzyme in mucopolipidosis II cells and in fibroblasts treated with NH_4Cl .

The enzyme has been shown to contain 5-15% Asn linked carbohydrate (Ledonne, et al., 1983, Arch.
10 Biochem. Biophys. 224: 186). The tissue form of this enzyme was shown to have ~52% high mannose and 48% complex type oligosaccharides. The high mannose type coeluted, on Bio-gel chromatography, with $\text{Man}_{8,9}\text{GlcNAc}$ while the complex type oligosaccharides were of two
15 categories containing 14 and 19-39 glucose units. Upon isoelectric focusing many forms of this enzyme are observed depending on the sources of the purified enzyme (tissue vs plasma form). However, upon
20 treatment with neuraminidase, a single band is observed (pI-5.1) indicating that this heterogeneity is due to different degrees of sialylation (Bishop & Desnick, 1981, J. Biol. Chem. 256: 1307). Initial efforts to express the full-length cDNA encoding α -Gal A involved using various prokaryotic expression
25 vectors (Hantzopoulos and Calhoun, 1987, Gene 57:159; Ioannou, 1990, Ph.D. Thesis, City University of New York). Although microbial expression was achieved, as evidenced by enzyme assays of intact E. coli cells and growth on melibiose as the carbon source, the human
30 protein was expressed at low levels and could not be purified from the bacteria. These results indicate that the recombinant enzyme was unstable due to the lack of normal glycosylation and/or the presence of endogenous cytoplasmic or periplasmic proteases.

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2.3. LYSOSOMAL ENZYMES: BIOSYNTHESIS AND TARGETING

5 Lysosomal enzymes are synthesized on membrane-bound polysomes in the rough endoplasmic reticulum. Each protein is synthesized as a larger precursor containing a hydrophobic amino terminal signal peptide. This peptide interacts with a signal recognition particle, an 11S ribonucleoprotein, and thereby initiates the vectorial transport of the nascent protein across the endoplasmic reticulum membrane into the lumen (Erickson, et al., 1981, J. Biol. Chem. 256: 11224; Erickson, et al., 1983, Biochem. Biophys. Res. Commun. 115: 275; Rosenfeld, et al., 1982, J. Cell Biol. 93: 135). Lysosomal enzymes are cotranslationally glycosylated by the en bloc transfer of a large preformed oligosaccharide, glucose-3, mannose-9, N-acetylglucosamine-2, from a lipid-linked intermediate to the Asn residue of a consensus sequence Asn-X-Ser/Thr in the nascent polypeptide (Kornfeld, R. & Kornfeld, S., 1985, Annu. Rev. Biochem. 54: 631). In the endoplasmic reticulum, the signal peptide is cleaved, and the processing of the Asn-linked oligosaccharide begins by the excision of three glucose residues and one mannose from the oligosaccharide chain.

25 The proteins move via vesicular transport, to the Golgi stack where they undergo a variety of posttranslational modifications, and are sorted for proper targeting to specific destinations: lysosomes, secretion, plasma membrane. During movement through the Golgi, the oligosaccharide chain on secretory and membrane glycoproteins is processed to the sialic acid-containing complex-type. While some of the oligosaccharide chains on lysosomal enzymes undergo similar processing, most undergo a different series of modifications. The most important modification is the

acquisition of phosphomannosyl residues which serve as an essential component in the process of targeting these enzymes to the lysosome (Kaplan, et al., 1977, Proc. Natl. Acad. Sci. USA 74: 2026). This recognition marker is generated by the sequential action of two Golgi enzymes. First, N-acetylglucosaminylphosphotransferase transfers N-acetylglucosamine-1-phosphate from the nucleotide sugar uridine diphosphate-N-acetylglucosamine to selected mannose residues on lysosomal enzymes to give rise to a phosphodiester intermediate (Reitman & Kornfeld, 1981, J. Biol. Chem. 256: 4275; Waheed, et al., 1982, J. Biol. Chem. 257: 12322). Then, N-acetylglucosamine-1-phosphodiester α -N-acetylglucosaminidase removes N-acetylglucosamine residue to expose the recognition signal, mannose-6-phosphate (Varki & Kornfeld, 1981, J. Biol. Chem. 256: 9937; Waheed, et al., 1981, J. Biol. Chem. 256: 5717).

Following the generation of the phosphomannosyl residues, the lysosomal enzymes bind to mannose-6-phosphate (M-6-P) receptors in the Golgi. In this way the lysosomal enzymes remain intracellular and segregate from the proteins which are destined for secretion. The ligand-receptor complex then exits the Golgi via a coated vesicle and is delivered to a prelysosomal staging area where dissociation of the ligand occurs by acidification of the compartment (Gonzalez-Noriega, et al., 1980, J. Cell Biol. 85: 839). The receptor recycles back to the Golgi while the lysosomal enzymes are packaged into vesicles to form primary lysosomes. Approximately, 5-20% of the lysosomal enzymes do not traffic to the lysosomes and are secreted presumably, by default. A portion of these secreted enzymes may be recaptured by the M-6-P receptor found on the cell surface and be internalized

and delivered to the lysosomes (Willingham, et al., 1981, Proc. Natl. Acad. Sci. USA 78: 6967).

Two mannose-6-phosphate receptors have been identified. A 215 kDa glycoprotein has been purified from a variety of tissues (Sahagian, et al., 1981, Proc. Natl. Acad. Sci. USA, 78: 4289; Steiner & Rome, 1982, Arch. Biochem. Biophys. 214: 681). The binding of this receptor is divalent cation independent. A second M-6-P receptor also has been isolated which differs from the 215 kd receptor in that it has a requirement for divalent cations. Therefore, this receptor is called the cation-dependent (M-6-P^{CD}) while the 215 kd one is called cation-independent (M-6-P^{CI}). The M-6-P^{CD} receptor appears to be an oligomer with three subunits with a subunit molecular weight of 46 kDa.

3. SUMMARY OF THE INVENTION

The present invention involves the production of large quantities of human α -Gal A by cloning and expressing the α -Gal A coding sequence in eukaryotic host cell expression systems. The eukaryotic expression systems, and in particular the mammalian host cell expression system described herein, provide for the appropriate cotranslational and posttranslational modifications required for proper processing, e.g., glycosylation, sialylation, phosphorylation, etc. and sorting of the expression product so that an active enzyme is produced. Also described is the expression of α -galactosidase A fusion proteins which are readily purified. These fusion proteins are engineered so that the α -galactosidase A moiety is readily cleaved from the fusion protein and recovered.

Using the methods described herein, the recombinant α -Gal A is secreted by the engineered host cells so that it is recovered from the culture medium in good yield. The α -Gal A produced in accordance with the invention may be used for a variety of ends, including but not limited to the treatment in Fabry Disease, the conversion of blood type B to O, or in any commercial process that involves the hydrolysis of α -D--galactosyl residues from glycoconjugates.

Further, this invention describes a method whereby proteins that are normally intracellularly targeted may be overexpressed and secreted from recombinant mammalian cell lines.

3.1. DEFINITIONS

As used herein, the following terms and abbreviations will have the indicated meaning:

	α -Galactosidase A	α -Gal A
20	α -N-AcetylGalactosaminidase	α -GalNAc
	base pair(s)	bp
	Chinese hamster ovary	CHO
	complementary DNA	cDNA
	counts per minute	cpm
25	deoxyribonucleic acid	DNA
	Dulbecco's Modified Eagle's Medium	DMEM
	fetal calf serum	FCS
	kilobase pairs	kb
	kilodalton	kDa
30	mannose-6-phosphate	M-6-P
	methotrexate	MTX
	4-methylumbelliferyl- α -D--galactoside	4-MU- α -Gal
	4-methyl-umbelliferyl- α -N-acetyl-galactosaminide	4-Mu- α -GalNAc
35	micrograms	μ g
	micrometer	μ m
	nanograms	ng

nanometer	nm
nucleotide	nt
p-nitrophenyl- α -N-Acetylgalactosaminide	pNP- α -GalNAc
5 polyacrylamide gel electrophoresis	PAGE
polymerase chain reaction	PCR
ribonucleic acid	RNA
sodium dodecyl sulfates	SDS
units	U

10 4. DESCRIPTION OF THE FIGURES

FIG. 1A-1C. Full-length human α -Gal cDNA sequence. N-terminal, cyanogen bromide (CB), and tryptic (T) peptide amino acid sequences obtained from peptide microsequencing are indicated by underlines. Differences from the sequence predicted from the cDNA are shown. The four putative N-glycosylation sites are denoted and the 3' termination signals are overlined.

20 FIG. 1D-1F. Alignment of amino acid sequences deduced from the full-length cDNAs encoding human α -Gal-NAc (α -Gal B), α -Gal, yeast Mel 1, and E. coli Mel A. Colons, identical residues; single dots, isofunctional amino acids; and boxes, identical residues in α -GalNAc, α -Gal, Mel 1 and/or Mel A. Gaps were introduced for optimal alignment. Numbered vertical lines indicate exon boundaries for α -Gal (Bishop, et al, 1988, Proc. Natl. Acad. Sci. USA 85: 3903-3907).

30 FIG. 1G. Construction of the α -Gal mammalian expression vector p91-AGA. The full-length cDNA was excised from plasmid pcDAG126, adapted by the addition of Eco RI linkers and subsequently cloned into the Eco RI site of expression vector p91023(B).

35 FIG. 2A-2B. Transient expression of human α -Gal in COS-1 cells. Maximum activity (U/mg) was reached
72

hours post-transfection in cells receiving the p91-AGA construct. No increase in α -Gal activity was observed in cells receiving no plasmid DNA nor in cells
5 receiving the p91 vector with the α -Gal cDNA in the reverse orientation.

FIG. 3A-3B. Serum effect on secretion of recombinant α -Gal by CHO DG5.3. Cells were plated in DMEM supplemented with the appropriate serum concentration
10 (FIG. 3A. Cells were plated in DMEM supplemented with 10% FCS. Following confluency (~4 days), the media was replaced with fresh DMEM supplemented with the appropriate serum concentration (FIG. 3B).

FIG. 4. High-level production of recombinant α -Gal in a hollow fiber bioreactor. The amount of fetal bovine serum required by this system for optimal cell-
15 growth and protein secretion could be decreased to about 1%.

FIG. 5. SDS-PAGE of each step of the α -Gal purification scheme. Lanes 1, 6, molecular weight
20 markers; lane 2, crude media; lane 3, affinity chromatography; lane 4, octyl-Sepharose chromatography; lane 5, superose 6 chromatography.

FIG. 6. Total cellular (lanes 1-4) and media (lanes 5-8) from control DG44 cells (lane 1,5), DG5
25 cells (lanes 2, 6), DG5.3 cells (lanes 3,7) and DG11 cells (lanes 4,8), labeled with [³⁵S]-methionine.

FIG. 7A-7C. Physicokinetic properties of recombinant α -Gal. Km towards the artificial
30 substrate 4-MU- α -D--galactopyranoside (FIG. 7A). Isoelectric point of recombinant and human plasma purified enzyme (FIG. 7B). pH optimum of the recombinant enzyme. (FIG. 7C).

FIG. 8A-8B. P-C₁₂STH degradation by CHO DG5.3 cells
35 overproducing human α -Gal. Rapid degradation of this substrate is observed by the accumulation of P-C₁₂SDH.

FIG. 9. Acquisition of disulfide bridges by recombinant α -Gal . CHO DG5.3 cells were labeled with [³⁵S]-methionine and chased for the indicated times. SDS-PAGE in the absence of a reducing agent reveals the formation of secondary structures through disulfide bond formation.

FIG. 10. Arrival of newly synthesized α -Gal to the Golgi network detected by the acquisition of Endo H resistant oligosaccharides.

FIG. 11. Secretion rate of recombinant α -Gal . CHO DG5.3 cells were labeled with [³⁵S]-methionine for 5 min and chased with cold methionine. Culture media aliquots were removed at the indicated times and immunoprecipitated with anti- α -Gal polyclonal antibodies.

FIG. 12. SDS-PAGE of culture media from DG44 (lane 1; control), DG5 (lane 2) and DG5.3 (lanes 3,4) cells labeled with [³⁵S]-methionine for 1 hour (lanes 1-3) and 24 hours (lane 4).

FIG. 13. Analysis of the carbohydrate moieties on recombinant α -Gal . CHO DG5.3 cells were labeled with [³⁵S]-methionine for 24 hours, the culture media collected and the recombinant enzyme immunoprecipitated. Aliquots were digested with endo D (lane 2), Endo H (lane 3), Endo F (lane 4), PNGase F (lane 5), Endo D and H (lane 6), Endo H and F (lane 7), and Endo H, F, and PNGase F (lane 8). Untreated samples (lanes 1, 9).

FIG. 14. Cellular (lanes 1,3) and secreted (lanes 2,4) forms of recombinant α -Gal treated with PNGase F (lanes 3,4). Controls (lanes 1,2).

FIG. 15. Effect of glycosylation inhibitors on the secretion of recombinant α -Gal.

FIG. 16. ^{32}P labelling of CHO DG44 (lanes 2, 3) and DG5.3 (lanes 1, 4). α -Gal was immunoprecipitated from cells (lanes 1, 2) and media (lanes 2, 3).

5 FIG. 17A-17D. QAE-Sephadex chromatography of endo H sensitive oligosaccharides of recombinant α -Gal. Untreated, dilute HCl treated, neuraminidase treated and alkaline phosphatase treated oligosaccharides.

10 FIG. 18. Endo H sensitive oligosaccharides of recombinant α -Gal chromatographed on M-6-P receptor. solid circles, peak minus 4, open circles, peak minus 2.

15 FIG. 19. Recombinant α -Gal chromatography on M-6-P receptor. DG5.3 cells labeled with [^{35}S]-methionine for 24 hours and media collected for chromatography. Solid circles, α -Gal activity; open boxes, total radioactivity.

20 FIG. 20A-20C. Recombinant and human α -Gal affinity chromatography on M-6-P receptor. Cells were labeled with [^{35}S]-methionine for 24 hours in the presence of NH_4Cl and the culture media were collected. DG5.3 secretions (FIG. 20A), MS914 secretions (FIG. 20B) and 293 secretions (FIG. 20C). Solid circles, α -Gal activity. Squares, total radioactivity. Open circles, M-6-P gradient used for elution.

25 FIG. 21. Uptake of recombinant α -Gal by Fabry fibroblasts. Cells were incubated for the indicated amounts of α -Gal for 6 hours. Open circles, α -Gal uptake, closed circles, uptake in the presence of 2mM M-6-P.

30 FIG. 22. Construction scheme of the α -Gal protein A fusion. The fusion was accomplished in two separate PCR reactions as described in Section 9.1.

35 FIG. 23. Nucleotide sequence of the protein A domain E, collagenase cleavage sequence and 3' α -Gal

sequence (23A). Schematic of the fusion construct showing the collagenase consensus in relation to the α -Gal and protein A domains (23B).

5 FIG. 24A-24B. Plasma clearance following intravenous administration to mice of the recombinant human secreted α -galactosidase A containing α 2,6-sialic acid residues (Δ -- Δ) and the non- α 2,6-sialylated glycoform (\bullet -- \bullet) upper graph, compared with
10 the plasma clearance of these glycoforms following treatment with acid phosphatase. Each point represents the average of the values from two independent injections. The $T_{1/2}$ values were estimated by extrapolation.

15 FIG. 25. Tissue distribution of different forms of recombinant human secreted α -Gal A following intravenous administration to mice. Each point represents the average of the values from two independent injections.

20 FIG. 26. DG5.3-1000Mx CHO Cells Contain Crystalline Structures of Overexpressed Human α -Gal A. Electron micrographs of DG5.3-1000Mx cells showing crystalline structures in single membrane-limited vacuoles (26A) and in vesicles, presumably in the
25 dilated trans-Golgi (26B; bar, 0.15 μ m and 0.10 μ m, respectively). 26C and D: Immunoelectron microscopic localization of human α -Gal A with 10 nm colloidal gold particles (bar, 0.31 μ m and 0.19 μ m, respectively). 26E: Electron micrograph of parental
30 dfhr DG44 cells (bar, 1.11 μ m); inset showing Golgi complex (arrows) in dfhr DG44 cells (bar, 0.5 μ m).

 FIG. 27A-27C. Aggregation of Purified Secreted α -Gal A is Enzyme Concentration and pH Dependent. 27A: Precipitation of α -Gal A (10 mg/ml) with
35 decreasing pH. Note that about 12% of incubated enzyme was precipitated at pH 6.0, the estimated pH of

the trans-Golgi Network (TGN). 27B: Turbidity of increasing concentrations of secreted α -Gal A at pH 5.0 (closed circles) and pH 7.0 (closed triangles) in the absence of Bovine Serum Albumin (BSA). As a control for non-specific precipitation with increasing protein concentration, secreted α -Gal A (1 mg/ml) was mixed with increasing BSA concentrations (0.1 to 10 mg/ml) at pH 5.0 (solid squares). 27C: SDS-PAGE of the supernatant and pellet fractions from mixtures of purified secreted α -Gal A (10 mg/ml) and BSA (2 mg/ml) incubated at decreasing pH values. Note that α -Gal A was precipitated with decreasing pH whereas the concentrations of soluble and precipitated BSA were essentially unchanged.

FIG. 28. Aggregation-Secretion Model for Selective Secretion of Human α -Gal A Overexpressed in CHO Cells. High level overexpression in CHO cells of human α -Gal A or other lysosomal enzymes normally targeted to lysosomes results in their selective secretion due to their aggregation and the resultant inaccessibility of their M6P signals. The enzymes undergo normal post-translational processing until arriving in the TGN, where the overexpressed enzyme undergoes protein-protein interactions and forms smaller soluble and larger particulate aggregates, due to lower pH of the TGN. The TGN becomes dilated with the overexpressed enzyme. Some aggregates and soluble enzyme with exposed M6P signals are trafficked to lysosomes, while the majority of aggregates whose M6P are not accessible are exocytosed by default via the constitutive secretory pathway. Also, decreased sulfation may occur as the tyrosines in enzyme aggregates destined for secretion are unavailable to sulfotransferase. The model may explain the selective secretion of other

other overexpressed proteins that normally are targeted to specific organelles.

5 5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the production of biologically active human α -Gal A involving cloning and expressing the nucleotide coding sequences for the enzyme in eukaryotic expression systems. Successful
10 expression and production of this purified, biologically active enzyme as described and exemplified herein is particularly significant for a number of reasons. For example, past efforts to express the full-length cDNA encoding α -Gal A using
15 various prokaryotic expression vectors resulted in expression of the enzyme, as evidenced by enzyme assays of intact microbial host cells and growth on melibiose as the carbon source; however, the human enzyme was expressed at low levels and could not be
20 purified from the bacteria. These results indicate that the recombinant enzyme expressed in microbial systems was unstable due to the lack of normal glycosylation and/or the presence of endogenous cytoplasmic or periplasmic proteases.

25 Efforts to express this enzyme in eukaryotic expression systems were equally difficult for different reasons. The α -Gal A is a lysosomal enzyme encoded by a "housekeeping" gene. The primary translation product is highly modified and processed,
30 requiring a complex series of events involving cleavage of a signal sequence, glycosylation, phosphorylation, and sialylation, which can be properly effected only by appropriate host cells. Moreover, since the expression product is destined for
35 the lysosome, which remains intracellular, it is quite surprising that the methods described herein allow for

the secretion of a properly processed, biologically active molecule.

The biologically active α -Gal A produced in accordance with the invention has a variety of uses, probably the most significant being its use in enzyme replacement therapy for the lysosomal storage disorder, Fabry disease. For example, the metabolic defect in cultured fibroblasts from Fabry disease can be corrected in vitro by the addition of exogenous α -Gal A into the culture medium. In addition, limited human trials have demonstrated the biochemical effectiveness of enzyme replacement to deplete the circulating substrate prior to vascular deposition. However, prior to the present invention, large quantities of biologically active, purified human α -Gal A could not be produced for use in replacement therapies. The α -Gal A produced in accordance with the invention also has a number of industrial uses, e.g., in any process involving the hydrolysis of α -D--galactosyl glycoconjugates, the conversion of blood group B to group O, etc., as described herein.

The invention is divided into the following sections solely for the purpose of description:

- (a) the coding sequence for α -Gal A; (b) construction of an expression vector which will direct the expression of the enzyme coding sequence;
- (c) transfection of appropriate host cells which are capable of replicating, translating and properly processing the primary transcripts in order to express a biologically active gene product; and
- (d) identification and/or purification of the enzyme so produced. Once a transformant is identified that expresses high levels of biologically active enzyme, the practice of the invention involves the expansion

and use of that clone in the production and purification of biologically active α -Gal A.

The invention is demonstrated herein, by way of
5 examples in which cDNAs of α -Gal A were cloned and expressed in a mammalian expression system. Modifications to the cDNA coding sequences which improve yield, and simplify purification without detracting from biological activity are also
10 described. Further, modifications to the host cells are described that allow for the expression of sialylated and asialylated glycoforms of the enzyme, both of which may be easily purified. Although the invention is described for α -Gal A, the methods and
15 modifications explained may be analogously applied to the expression of other secreted proteins, and in particular, other lysosomal enzymes, including but not limited to α -N-acetylgalactosaminidase, and acid sphingomyelinase.

20 Various aspects of the invention are described in more detail in the subsections below and in the examples that follow.

5.1. THE α -GAL A CODING SEQUENCE

25 The nucleotide coding sequence and deduced amino acid sequence for α -Gal A is depicted in FIG. 1A-1C. This nucleotide sequence, or fragments or functional equivalents thereof, may be used to generate recombinant DNA molecules that direct the expression
30 of the enzyme product, or functionally active peptides or functional equivalents thereof, in appropriate host cells.

Due to the degeneracy of the nucleotide coding sequence, other DNA sequences which encode sub-
35 stantially the same amino acid sequences as depicted in FIG. 1A-1C may be used in the practice of the

invention for the cloning and expression of α -Gal A. Such alterations include deletions, additions or substitutions of different nucleotide residues
5 resulting in a sequence that encodes the same or a functionally equivalent gene product. The gene product may contain deletions, additions or substitutions of amino acid residues within the sequence, which result in a silent change thus
10 producing a bioactive product. Such amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, the amphipathic nature of the residues involved and/or on the basis of crystallographic data.
15 For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; amino acids with uncharged polar head groups having similar hydrophilicity values include the following: leucine,
20 isoleucine, valine; glycine, alanine; asparagine, glutamine; serine, threonine; phenylalanine, tyrosine.

The coding sequences for α -Gal A may be conveniently obtained from genetically engineered microorganisms or cell lines containing the enzyme
25 coding sequences, such as the deposited embodiments described herein. Alternatively, genomic sequences or cDNA coding sequences for these enzymes may be obtained from human genomic or cDNA libraries. Either genomic or cDNA libraries may be prepared from DNA
30 fragments generated from human cell sources. The fragments which encode α -Gal A may be identified by screening such libraries with a nucleotide probe that is substantially complementary to any portion of the sequence depicted in FIG. 1A-1C. Indeed, sequences
35 generated by polymerase chain reaction can be ligated to form the full-length sequence. Although portions

of the coding sequences may be utilized, full length clones, i.e., those containing the entire coding region for α -Gal A, may be preferable for expression.

5 Alternatively, the coding sequences depicted in FIG. 1A-1C may be altered by the addition of sequences that can be used to increase levels of expression and/or to facilitate purification. For example, as demonstrated in the working embodiments described herein, the α -Gal

10 A coding sequence was modified by the addition of the nucleotide sequence encoding the cleavage site for collagenase followed by the Staphylococcal Protein A. Expression of this chimeric gene construct resulted in a fusion protein consisting of α -Gal A- the

15 collagenase substrate -- Protein A. This fusion protein was readily purified using an IgG column which binds to the Protein A moiety. Unfused α -Gal A was released from the column by treatment with collagenase which cleaved the α -Gal A from the Protein A moiety

20 bound to the column. Other enzyme cleavage substrates and binding proteins can be engineered into similar constructs for the production of α -Gal A which can be readily purified and released in its biologically active form.

25 Techniques well-known to those skilled in the art for the isolation of DNA, generation of appropriate restriction fragments, construction of clones and libraries, and screening recombinants may be used. For a review of such techniques, see, for example,

30 Sambrook, et al., 1989, Molecular Cloning A Laboratory Manual, 2nd Ed. Cold Spring Harbor Press, N.Y., Chapters 1-18.

In an alternate embodiment of the invention, the coding sequence of FIG. 1A-1C could be synthesized in

35 whole or in part, using chemical methods well-known in the art. See, for example, Caruthers, et. al., 1980,

Nuc. Acids Res. Symp. Ser. 7: 215-233; Crea & Horn, 1980, Nuc. Acids Res. 9(10): 2331; Matteucchi & Carruthers, 1980, Tetrahedron Letters 21: 719; and
5 Chow and Kempe, 1981, Nuc. Acids Res. 9(12): 2807-2817.

Alternatively, the protein itself could be produced using chemical methods to synthesize the amino acid sequence depicted in FIG. 1A-1C in whole or
10 in part. For example, peptides can be synthesized by solid phase techniques, cleaved from the resin and purified by preparative high performance liquid chromatograph. (E.g., see, Creighton, 1983, Proteins, Structures and Molecular Principles, W.H. Freeman &
15 Co., N.Y. pp. 50-60). The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure; see Creighton, 1983, Proteins, Structures and Molecular Principles, W. H. Freeman & Co., N.Y.,
20 pp. 34-49).

Human α -Gal A is a homodimeric glycoprotein. The full-length α -Gal A cDNA predicts a mature subunit of 398 amino acids. The amino acid sequence has an overall homology of about 50% with human α -N-
25 acetylgalactosaminidase (α -Gal B). Homology searches with computerized data bases revealed short regions of α -Gal A homology with the yeast Mel 1 and the E. coli Mel A amino acid sequences (see FIG. 1D-1F). It is likely that these conserved regions are important for
30 enzyme conformation, stability, subunit association and/or catalysis. Thus, it is preferred not to alter such conserved regions. However, certain modifications in the coding sequence may be advantageous. For example, the four N-linked
35 glycosylation consensus sequences could be selectively obliterated, thereby altering the glycosylation of the

enzyme and affecting phosphorylation, sialylation, sulfation, etc. Such modified enzymes may have altered clearance properties and targeting when
5 injected into Fabry patients.

Oligosaccharide modifications may be useful in the targeting of α -Gal A for effective enzyme therapy. Some examples of such modifications are described in more detail infra. Previous studies demonstrated that
10 the plasma glycoform of α -Gal A, which is more highly sialylated than the splenic glycoform, was more effective in depleting the toxic accumulated circulating substrate from Fabry patients (Desnick et al., 1977, Proc. Natl. Acad. Sci. USA 76:5326-5330).
15 Studies characterizing the purified splenic and plasma glycoforms of the enzyme revealed differences only in their oligosaccharide moieties (Desnick et al., 1977, Proc. Natl. Acad. Sci. USA 76:5326-5330). Thus, efforts to target the recombinant enzyme for effective
20 treatment of Fabry disease may be enhanced by modification of the N-glycosylation sites.

Also, the 5' untranslated and coding regions of the nucleotide sequence could be altered to improve the translational efficiency of the α -Gal A mRNA. For
25 example, substitution of a cytosine for the guanosine in position +4 of the α -Gal A cDNA could improve the translational efficiency of the α -Gal A mRNA 5- to 10-fold (Kozak, 1987, J. Mol. Biol. 196:947-950).

In addition, based on X-ray crystallographic
30 data, sequence alterations could be undertaken to improve protein stability, e.g., introducing disulfide bridges at the appropriate positions, and/or deleting or replacing amino acids that are predicted to cause protein instability. These are only examples of
35 modifications that can be engineered into the α -Gal A enzyme to produce a more active or stable protein,

more enzyme protein, or even change the catalytic specificity of the enzyme.

5 5.2. PRODUCTION OF RECOMBINANT α -Gal A

In order to express a biologically active α -Gal A, the coding sequence for the enzyme, a functional equivalent, or a modified sequence, as described in Section 5.1., supra, is inserted into an appropriate eukaryotic expression vector, i.e., a vector which contains the necessary elements for transcription and translation of the inserted coding sequence in appropriate eukaryotic host cells which possess the cellular machinery and elements for the proper processing, i.e., signal cleavage, glycosylation, phosphorylation, sialylation, and protein sorting. Mammalian host cell expression systems are preferred for the expression of biologically active enzymes that are properly folded and processed; when administered in humans such expression products should exhibit proper tissue targeting and no adverse immunological reaction.

25 5.2.1. CONSTRUCTION OF EXPRESSION VECTORS AND PREPARATION OF TRANSFECTANTS

Methods which are well-known to those skilled in the art can be used to construct expression vectors containing the α -Gal A coding sequence and appropriate transcriptional/translational control signals. These methods include in vitro recombination/genetic recombination. See, for example, the techniques described in Maniatis et al., 1982, Molecular Cloning A Laboratory Manual, Cold spring Harbor Laboratory, N.Y., Chapter 12.

35 A variety of eukaryotic host-expression systems may be utilized to express the α -Gal A coding sequence. Although prokaryotic systems offer the

distinct advantage of ease of manipulation and low cost of scale-up, their major drawback in the expression of α -Gal A is their lack of proper post-translational modifications of expressed mammalian proteins. Eukaryotic systems, and preferably mammalian expression systems, allow for proper modification to occur. Eukaryotic cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, phosphorylation, and, advantageously secretion of the gene product should be used as host cells for the expression of α -Gal A. Mammalian cell lines are preferred. Such host cell lines may include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, -293, WI38, etc. Alternatively, eukaryotic host cells which possess some but not all of the cellular machinery required for optional processing of the primary transcript, and/or post-translational processing and/or secretion of the gene product may be modified to enhance the host cell's processing capabilities. For example, a recombinant nucleotide sequence encoding a peptide product that performs a processing function the host cell had not previously been capable of may be engineered into the host cell line. Such a sequence may either be co-transfected into the host cell along with the gene of interest, or included in the recombinant construct encoding the gene of interest. Alternatively, cell lines containing this sequence may be produced which are then transfected with the gene of interest.

Appropriate eukaryotic expression vectors should be utilized to direct the expression of α -Gal A in the host cell chosen. For example, at least two basic approaches may be followed for the design of vectors on SV40. The first is to replace the SV40 early

region with the gene of interest while the second is to replace the late region (Hammarskjold, et al., 1986, Gene 43: 41). Early and late region replacement
5 vectors can also be complemented in vitro by the appropriate SV40 mutant lacking the early or late region. Such complementation will produce recombinants which are packaged into infectious capsids and which contain the α -Gal A gene. A
10 permissive cell line can then be infected to produce the recombinant protein. SV40-based vectors can also be used in transient expression studies, where best results are obtained when they are introduced into COS (CV-1, origin of SV40) cells, a derivative of CV-1
15 (green monkey kidney cells) which contain a single copy of an origin defective SV40 genome integrated into the chromosome. These cells actively synthesize large T antigen (SV40), thus initiating replication from any plasmid containing an SV40 origin of
20 replication.

In addition to SV40, almost every molecularly cloned virus or retrovirus may used as a cloning or expression vehicle. Viral vectors based on a number of retroviruses (avian and murine), adenoviruses,
25 vaccinia virus (Cochran, et al., 1985, Proc. Natl. Acad. Sci. USA 82: 19) and polyoma virus may be used for expression. Other cloned viruses, such as JC (Howley, et al., 1980, J. Virol 36: 878), BK and the human papilloma viruses (Heilman, et al., 1980, J.
30 Virol 36: 395), offer the potential of being used as eukaryotic expression vectors. For example, when using adenovirus expression vectors the α -Gal A coding sequence may be ligated to an adenovirus transcription/translation control complex, e.g., the
35 late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus

- 26 -

genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the human enzyme in infected hosts (e.g., see Logan & Shenk, 1984, Proc. Natl. Acad. Sci. (USA) 81: 3655-3659). Alternatively, the vaccinia virus 7.5K promoter may be used. (e.g., see, Mackett et al., 1982, Proc. Natl. Acad. Sci. (USA) 79: 7415-7419; Mackett et al., 1984, J. Virol. 49: 857-864; Panicali et al., 1982, Proc. Natl. Acad. Sci. 79: 4927-4931). Of particular interest are vectors based on bovine papilloma virus (Sarver, et al., 1981, Mol. Cell. Biol. 1: 486). These vectors have the ability to replicate as extrachromosomal elements. Shortly after entry of this DNA into mouse cells, the plasmid replicates to about 100 to 200 copies per cell. Transcription of the inserted cDNA does not require integration of the plasmid into the host's chromosome, thereby yielding a high level of expression. These vectors can be used for stable expression by including a selectable marker in the plasmid, such as the neo gene. High level expression may also be achieved using inducible promoters such as the metallothionine IIA promoter, heat shock promoters, etc.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the α -Gal A or DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription

terminators, polyadenylation sites, etc.), and a selectable marker. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11: 223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48: 2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22: 817) genes can be employed in tk^r, hgp^rt^r or ap^rt^r cells respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77: 3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78: 1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78: 2072; neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150: 1); and hyg^r, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30: 147) genes. Recently, additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman & Mulligan, 1988, Proc. Natl. Acad. Sci. USA 85: 8047); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue L., 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.).

Alternative eukaryotic expression systems which may be used to express the α -Gal A enzymes are yeast transformed with recombinant yeast expression vectors
5 containing the α -Gal A coding sequence; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the α -Gal A coding sequence; or plant cell systems infected with recombinant virus expression vectors (e.g.,
10 cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing the α -Gal A coding sequence.

In yeast, a number of vectors containing
15 constitutive or inducible promoters may be used. For a review see, Current Protocols in Molecular Biology, Vol. 2, 1988, Ed. Ausubel et al., Greene Publish. Assoc. & Wiley Interscience, Ch. 13; Grant et al., 1987, Expression and Secretion Vectors for Yeast, in
20 Methods in Enzymology, Eds. Wu & Grossman, 31987, Acad. Press, N.Y., Vol. 153, pp.516-544; Glover, 1986, DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3; and Bitter, 1987, Heterologous Gene Expression in Yeast, Methods in Enzymology, Eds. Berger & Kimmel,
25 Acad. Press, N.Y., Vol. 152, pp. 673-684; and The Molecular Biology of the Yeast Saccharomyces, 1982, Eds. Strathern et al., Cold Spring Harbor Press, Vols. I and II. For complementation assays in yeast, cDNAs for α -Gal A may be cloned into yeast episomal plasmids
30 (YE_p) which replicate autonomously in yeast due to the presence of the yeast 2μ circle. The cDNA may be cloned behind either a constitutive yeast promoter such as ADH or LEU2 or an inducible promoter such as GAL (Cloning in Yeast, Chpt. 3, R. Rothstein In: DNA
35 Cloning Vol.11, A Practical Approach, Ed. DM Glover, 1986, IRL Press, Wash., D.C.). Constructs may contain

the 5' and 3' non-translated regions of the cognate α -Gal A mRNA or those corresponding to a yeast gene.

YEp plasmids transform at high efficiency and the plasmids are extremely stable. Alternatively, vectors may be used which promote integration of foreign DNA sequences into the yeast chromosome.

In cases where plant expression vectors are used, the expression of the α -Gal A coding sequence may be driven by any of a number of promoters. For example, viral promoters such as the 35S RNA and 19S RNA promoters of CaMV (Brisson et al., 1984, Nature 310:511-514), or the coat protein promoter of TMV (Takamatsu et al., 1987, EMBO J. 6:307-311) may be used; alternatively, plant promoters such as the small subunit of RUBISCO (Coruzzi et al., 1984, EMBO J. 3:1671-1680; Broglie et al., 1984, Science 224:838-843); or heat shock promoters, e.g., soybean hsp17.5-E or hsp17.3-B (Gurley et al., 1986, Mol. Cell. Biol. 6:559-565) may be used. These constructs can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors; direct DNA transformation; microinjection, electroporation, etc. For reviews of such techniques see, for example, Weissbach & Weissbach, 1988, Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp. 421-463; and Grierson & Corey, 1988, Plant Molecular Biology, 2d Ed., Blackie, London, Ch. 7-9.

An alternative expression system which could be used to express α -Gal A is an insect system. In one such system, Autographa californica nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in Spodoptera frugiperda cells. The α -Gal A sequence may be cloned into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an

AcNPV promoter (for example the polyhedrin promoter). Successful insertion of the coding sequence will result in inactivation of the polyhedrin gene and
5 production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect Spodoptera frugiperda cells in which the inserted gene is expressed. (E.g., see Smith et
10 al., 1983, J. Viol. 46:584; Smith, U.S. Patent No. 4,215,051).

5.2.2. IDENTIFICATION OF TRANSFECTANTS
OR TRANSFORMANTS EXPRESSING THE
 α -Gal A PRODUCT

15 The host cells which contain the α -Gal A coding sequence and which express the biologically active gene product may be identified by at least four general approaches: (a) DNA-DNA or DNA-RNA hybridization; (b) the presence or absence of "marker"
20 gene functions; (c) assessing the level of transcription as measured by the expression of α -Gal A mRNA transcripts in the host cell; and (d) detection of the gene product as measured by immunoassay or by its biological activity.

25 In the first approach, the presence of the α -Gal A coding sequence inserted in the expression vector can be detected by DNA-DNA or DNA-RNA hybridization using probes comprising nucleotide sequences that are homologous to the α -Gal A coding sequence
30 substantially as shown in FIG. 1A-1C, or portions or derivatives thereof.

In the second approach, the recombinant expression vector/host system can be identified and selected based upon the presence or absence of certain
35 "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, resistance to

methotrexate, transformation phenotype, occlusion body formation in baculovirus, etc.). For example, if the α -Gal A coding sequence is inserted within a marker gene sequence of the vector, recombinants containing the α -Gal A coding sequence can be identified by the absence of the marker gene function. Alternatively, a marker gene can be placed in tandem with the α -Gal A sequence under the control of the same or different promoter used to control the expression of the α -Gal A coding sequence. Expression of the marker in response to induction or selection indicates expression of the α -Gal A coding sequence.

In the third approach, transcriptional activity for the α -Gal A coding region can be assessed by hybridization assays. For example, RNA can be isolated and analyzed by Northern blot using a probe homologous to the α -Gal A coding sequence or particular portions thereof substantially as shown in FIG. 1A-1C. Alternatively, total nucleic acids of the host cell may be extracted and assayed for hybridization to such probes.

In the fourth approach, the expression of the α -Gal A protein product can be assessed immunologically, for example by Western blots, immunoassays such as radioimmuno-precipitation, enzyme-linked immunoassays and the like. The ultimate test of the success of the expression system, however, involves the detection of the biologically active α -Gal A gene product. Where the host cell secretes the gene product, the cell free media obtained from the cultured transfectant host cell may be assayed for α -Gal A activity. Where the gene product is not secreted, cell lysates may be assayed for such activity. In either case, a number of assays can be used to detect α -Gal A activity including but not limited to: (a) assays employing

the synthetic fluorogenic or chromogenic α -D-galactosides such as 4-methylumbelliferyl- α -D-galactopyranoside (Desnick et al., 1973, J. Lab. Clin. Invest. 81:157); (b) assays employing the radiolabeled or fluorescent labeled natural substrates such as tritiated globotriaosyl ceramide or pyrene-dodecanoyl-sphingosine-trihexoside (Bishop and Desnick, 1981, J. Biol. Chem. 256:1307); and (c) assays employing X- α -gal.

5.2.3. PURIFICATION OF THE α -GAL A GENE PRODUCT

Once a clone that produces high levels of biologically active α -Gal A is identified, the clone may be expanded and used to produce large amounts of the enzyme which may be purified using techniques well-known in the art including, but not limited to immunoaffinity purification, chromatographic methods including high performance liquid chromatography and the like. Where the enzyme is secreted by the cultured cells, α -Gal A may be readily recovered from the culture medium.

As demonstrated in the working examples described infra, recombinant α -Gal A was purified from the crude media by affinity chromatography on α -GalNH₂-C₁₂-Sepharose followed by hydrophobic chromatography on Octyl Sepharose and gel filtration on a 100 cm Superose 6 column. The recombinant enzyme was essentially homogeneous following the gel filtration step and was >98% pure as judged by SDS-PAGE.

Human recombinant α -Gal A was purified to homogeneity from the media of the CHO cell line, DG5.3, which was shown to secrete most of the recombinant enzyme. The culture media from this clone was highly enriched for α -Gal A when serum-free medium was used, constituting greater than 95% of the total

extracellular protein. Thus, purification to homogeneity could be accomplished in only three chromatographic steps. Over half a gram of enzyme was produced in three months and from a portion of this, 280 mg was purified with a yield of 80% using only laboratory-scale equipment. Notably, the recombinant enzyme had full enzymatic activity with a specific activity equal to that of the previously purified human enzyme (Bishop, et al., 1978, *Biochim. Biophys. Acta.* 525: 399; Bishop and Desnick, 1981, *J. Biol. Chem.* 256:1307). The recombinant enzyme was able to recognize and effectively cleave an analog of the natural substrate, globotriaosylceramide.

Where the α -Gal A coding sequence is engineered to encode a cleavable fusion protein, the purification of α -Gal A may be readily accomplished using affinity purification techniques. In the working examples described infra, a collagenase cleavage recognition consensus sequence was engineered between the carboxy terminus of α -Gal A and protein A. The resulting fusion protein was readily purified using an IgG column that bound the protein A moiety. Unfused α -Gal A was readily released from the column by treatment with collagenase.

In particular, the overlap extension method (Ho, et al., 1989, *Gene* 77: 51; Kadowaki, et al., 1989, *Gene* 76: 161) was used to fuse the full-length α -Gal A cDNA to the protein A domain E of *Staphylococcus aureus*. Following transfection by electroporation, the α -Gal A activity in COS-1 cell extracts was increased 6 to 7-fold. In addition, the transfected cells secreted significant amounts of the fusion protein into the culture media (400 U/ml). The secreted fusion protein was rapidly purified by a single IgG affinity purification step. The

engineering of a collagenase cleavage recognition
consensus sequence between these two polypeptides
facilitated the cleavage of the fusion protein so that
5 the purified human α -Gal A polypeptide could be
readily separated from the protein A domain by a
second IgG purification step. Of interest was the
fact that the fusion construct retained α -Gal
activity, presumably indicating that the enzyme
10 polypeptide formed the active homodimeric
configuration even though the carboxy terminus was
joined to an additional 56 residues of the protein A
domain. Since COS-1 cells transfected with an α -Gal A
construct exhibit similar levels of expression and
15 distribution between cells and media it appears that
the protein A domain does not interfere with either
the folding or the proper processing of this lysosomal
enzyme. Furthermore, the presence of the dimerized α -
Gal A polypeptide did not inhibit the binding of the
20 protein A domain to the IgG affinity column. The
insertion of the four residue collagenase cleavage
recognition sequence between the α -Gal A and protein A
polypeptides permitted cleavage of the fusion protein
leaving only two of the collagen residues on each of
25 the peptides.

The ease of cDNA construction using the
polymerase chain reaction, transfection and
purification of the expressed protein permits the
isolation of small, but sufficient amount of α -Gal A
30 for characterization of the enzyme's physical and
kinetic properties. Using site-directed mutagenesis
or naturally occurring mutant sequences, this system
provides a reasonable approach to determine the
effects of the altered primary structure on the
35 function of the protein. Fusion constructs with the
protein A domain E preceding the amino terminus and

the following the carboxy terminus may also be engineered to evaluate which fusion construct will interfere the least, if at all, with the protein's biologic function and the ability to bind IgG.

Using this aspect of the invention, any cleavage site or enzyme cleavage substrate may be engineered between the α -Gal A sequence and a second peptide or protein that has a binding partner which could be used for purification, e.g., any antigen for which an immunoaffinity column can be prepared.

5.2.4. CHARACTERIZATION OF THE RECOMBINANT ENZYME

The purified recombinant enzyme produced in the mammalian expression systems described herein (e.g., the CHO expression system), had molecular weight, pH optimum, km and isoelectric point values which were essentially identical to those of the enzyme purified from the human plasma (Bishop, et al., 1978, Biochim. Biophys. Acta. 525: 399; Bishop and Desnick, 1981, J. Biol. Chem. 256:1307). Analysis of the carbohydrate moieties on this enzyme revealed the presence of three oligosaccharide chains on the α -Gal A polypeptide. These chains were a mixture of complex, hybrid and high-mannose types as evidenced by endoglycosidase and QAE SephadexTM studies. Most importantly, the recombinant enzyme was also similar to the native plasma form of α -Gal A in having terminal sialic acid moieties (Bishop & Desnick, 1981, J Biol. Chem. 256: 1307). In the limited clinical trial described supra, the plasma form of the enzyme was shown to be more effective in degrading circulating GbOse₃Cer than the splenic form. Therefore, the recombinant enzyme or a modified recombinant enzyme, including but not limited to modifications of its carbohydrate chains or amino acid sequence, may be the most appropriate form for

enzyme replacement therapy of Fabry disease. Indeed, the saturable uptake of recombinant α -Gal A by Fabry and normal fibroblasts is demonstrated in the examples
5 herein, and is shown to be specifically inhibited by 2 mM mannose-6-phosphate.

In addition, the CHO expression system described herein has great promise for studies of the cell biology of lysosomal biogenesis and glycohydrolase
10 processing. Light microscopy revealed highly vacuolated cytoplasm in the DG5.3 CHO cells suggesting a proliferation of lysosomal membranes and offering the potential for analysis of lysosomal biogenesis. Preliminary studies have indicated that the
15 recombinant enzyme is synthesized very rapidly, exits the endoplasmic reticulum in 5-10 min following its synthesis and is secreted 45-60 min later. These fast kinetics of recombinant α -Gal A biosynthesis allow for interesting studies involving lysosomal enzyme
20 biosynthesis and offer a methodology that, to date, is only rivaled by viral systems. In fact, recombinant α -Gal A is synthesized so rapidly that a single radioactive pulse of 3 min is sufficient to label enough enzyme for these studies. The unexpectedly
25 specific secretion of only the overproduced recombinant α -Gal A and not other lysosomal enzymes appears analogous to "gene dosage-dependant secretion" described by Rothman, et al. (Stevens et al., 1986, J. Cell Biol. 102:1551; Rothman et al., 1986, Proc. Natl.
30 Acad. Sci. USA 83:3248) and poses interesting questions which can be evaluated in this system.

5.2.5. MODIFIED GLYCOFORMS OF RECOMBINANT α -Gal A FOR ENZYME THERAPY IN FABRY DISEASE

35 Initial experiments to assess the clearance kinetics and tissue distribution of recombinant α -Gal

A in mice revealed 50% targeting to the liver with the remaining enzyme being distributed to many other tissues including significant targeting to kidney, heart and skin. While this distribution is similar to that previously observed for the plasma form of human α -Gal A in mice, it may be appropriate to modify the enzyme for altered tissue targeting. Modifications of the recombinant α -Gal A to enhance tissue targeting including selective deglycosylation of the complex and high mannose carbohydrate moieties covalently attached to the recombinant enzyme. In particular, the invention includes modification of host cells that allow for expression of sialylated and asialylated glycoforms of the enzyme, both of which may be easily purified (see Section 9 *infra*). For example, when using CHO cells to express α -Gal A, the CHO cells may be co-transfected with sialyl-transferase gene construct that supplies the missing function to the CHO cell in order to express the sialylated glycoform of α -Gal A.

Alternatively, sequential deglycosylation to various glycoforms for use in the treatment of Fabry disease. Such modifications have proven to be important in effectively targeting β -glucocerebrosidase to macrophages in the treatment of Gaucher disease (Barton, N.W., et al., 1990, Proc. Natl. Acad. Sci. USA 87: 1913). In this case, placenta derived β -glucocerebrosidase was sequentially treated with neuraminidase, β -galactosidase and N- β -acetylglucosaminidase to expose terminal mannose residues for uptake by the mannose receptor of these cells (Stahl, et al., *in* The Molecular Basis of Lysosomal Disorders, Barranger, J.A. and Brady, R.O. eds., 1984 Academic Press, NY pp. 209-218).

Modifications to human recombinant α -Gal A included in the scope of this invention include, but are not limited to, sequential deglycosylation by
5 neuraminidase to expose terminal galactose; β -galactosidase treatment to expose N- β -acetylglucosaminyl residues; and N- β -acetylglucosaminidase treatment to expose mannose residues for specific targeting and uptake by various
10 cell types. The sequentially deglycosylated recombinant α -Gal A glycoforms may be analyzed by determining the clearance kinetics and tissue distribution of each of the radiolabeled glycoforms following intravenous administration in mice and
15 monkeys.

Deglycosylation of recombinant α -Gal A may be accomplished in a number of ways. The general methods of sequential treatment by exo-glycosidases which may be used are essentially those previously described
20 (Murray, G. J., 1987, Meth. Enzymol, 149: 25). For example, terminal sialic acid residues can be removed by treatment with neuraminidase covalently bound to agarose; e.g., type VI neuraminidase attached to agarose (SIGMA Chemical Co., St. Louis, MO) may be
25 used at 40 U/g to treat 100 mg α -Gal A with 8 units of conjugated neuraminidase at pH 5.0 for 4 hour at 37°C. The conjugated neuraminidase can be removed by centrifugation. Similarly, β -galactosidase (3 Units per 100 mg α -Gal A) purified from Streptococcus
30 pneumoniae may be used to remove terminal galactose residues. Finally, jack bean N- β -acetylglucosaminidase (SIGMA Chemical Co., St. Louis, MO) can be used; e.g., 3×10^6 units can be mixed with each 100 mg aliquot of the recombinant α -Gal A for
35 four hours at 37°C. At each step, the recombinant enzyme can be rapidly purified free of deglycosylating

enzymes and free carbohydrate by purification over the α -galactosylamine-SepharoseTM affinity column.

For the analysis of the in vivo fate of the
5 various glycoforms, including plasma clearance
kinetics and tissue distribution studies, the
recombinant α -Gal A may be labeled prior to
modification. For example, the recombinant α -Gal A
can be radiolabelled by growth in the CHO DG5.3 cell
10 line in the presence of 50 μ Ci/ml [³⁵S]methionine
(>1000 Ci/mole) for 24 hours. The secreted
radiolabeled enzyme can be purified from the harvested
media by α -galactosylamine-Sepharose affinity
chromatography as previously described. Essentially
15 100% of the radiolabelled protein secreted by these
cells is α -Gal A which can then be used for the
sequential generation of the glycoforms.

5.3. USES OF THE RECOMBINANT α -Gal A

20 The purified products obtained in accordance with
the invention may be advantageously utilized for
enzyme replacement therapy in patients with the
lysosomal storage disorder, Fabry Disease.
Alternatively, the purified products obtained in
25 accordance with the invention may be used in vitro to
modify α -D--galacto-glyconjugates in a variety of
processes; e.g., to convert blood group B erythrocytes
to blood group O; in commercial processes requiring
the conversion of sugars such as raffinose to sucrose
30 or melibiose to galactose and glucose; etc. These are
discussed in more detail in the subsections below.

5.3.1. α -Gal A ENZYME THERAPY IN FABRY DISEASE

35 Among the inborn errors of metabolism, studies of
patients with lysosomal storage disorders have
provided basic understanding of the biology of the

lysosomal apparatus and its hydrolases, their biosynthesis and processing (Rosenfeld, et al., 1982, J. Cell Biol. 93: 135; Lemansky, et al., 1984, J. Biol. Chem. 259: 10129), the mechanisms of their transport to the lysosomes (Neufeld, et al., 1975, Ann. Rev. Biochem. 44: 357; Sly et al., 1982, J. Cell Biochem. 18:67; Kornfeld, S., 1986, J. Clin. Invest. 77: 1), and their cofactor requirements (Verheijen, et al., 1985. Eur. J. Biochem. 149: 315; d'Azzo, et al., 1982, Eur. J. Biochem. 149: 315; Mehl, et al., 1964, Physiol. Chem. 339: 260; Conzelman, et al., 1978, Proc. Natl. Acad. Sci. USA 75: 3979). Of the over 30 lysosomal storage disorders, Fabry disease is an ideal candidate for the application of the recombinant DNA techniques described herein to evaluate and utilize various therapeutic approaches in model systems, as well as to correlate the effects of site-specific changes on enzyme structure and function. The disease has no central nervous system involvement; thus, the blood/brain barrier does not present an obstacle to enzyme replacement therapy. The defective enzyme, α -Gal A, is a homodimer (Bishop & Desnick, 1981, J. Biol. Chem. 256: 1307), in contrast to some lysosomal enzymes which have different subunits such as β -hexosaminidase A (Mahuran, et al., 1982, Proc. Natl. Acad. Sci. USA 79: 1602); therefore, only a single gene product must be obtained. The metabolic defect in cultured fibroblasts from Fabry disease has been corrected in vitro by the addition of exogenous enzyme into the culture medium (Cline, et al., 1986, DNA 5: 37). Also, atypical variants with Fabry disease have been identified, these males are clinically asymptomatic, having sufficient residual α -Gal A activity (3 to 10%) to protect them from the major morbid manifestations of the disease (Lemansky, et

al., 1987, J. Biol. Chem. 262:2062; Clarke, et al.,
1971, N. Engl. J. Med. 284: 233; Romeo, et al., 1975,
Biochem. Genet. 13: 615; Bishop, et al., 1981, Am. J.
5 Hum. Genet. 71: 217A; Bach, et al., 1982, Clin. Genet.
21: 59; and, Kobayashi, et al., 1985, J. Neurol. Sci.
67: 179). Finally, as noted above, limited human
trials have demonstrated the biochemical effectiveness
of enzyme replacement to deplete the circulating
10 substrate prior to vascular deposition as well as the
absence of immunologic complications (Brady, et al.,
1973, N. Engl. J. Med. 289: 9; Desnick, et al., 1979,
Proc. Natl. Acad. Sci. USA 76:5326; Bishop, et al.,
1981, Enzyme Therapy XX: In: Lysosomes and Lysosomal
15 Storage Diseases, Callahan, J.W. and Lowden, J.A.,
(eds.), Raven Press, New York, pp. 381; Desnick, et
al., 1980, Enzyme Therapy XVII: In: Enzyme Therapy in
Genetic Disease: 2, Desnick, R.J. (ed.), Alan, R.
Liss, Inc., New York, pp. 393).

20 In these studies, both splenic and plasma
isoforms of the α -Gal A enzyme were administered
intravenously. The circulating half-life of the
splenic isozyme was about 10 min whereas that for the
plasma isozyme was approximately 70 min. After each
25 dose of the splenic isozyme, the concentration of the
accumulated circulating substrate decreased maximally
in 15 min. In contrast, injection of the plasma
isozyme decreased circulating substrate levels
gradually over 36 - 72 hours. Since the secreted form
30 of the recombinant α -Gal A appears to be similar to
the plasma isozyme, the secreted form of the
recombinant enzyme could be effective for the long
term depletion and control of circulating substrate
levels.

35 The dose of the partially purified plasma and
splenic isozymes administered in the above clinical

5 trials was 2000 U/kg body weight, or a dose equivalent to giving 1 $\mu\text{g}/\text{kg}$ of pure enzyme. Since this dose proved effective in reducing the level of circulating
substrate, a similar dose of the recombinant enzyme
should have a similar effect. However, the
recombinant enzyme could be administered at a dosage
ranging from 0.1 $\mu\text{g}/\text{kg}$ to about 10 mg/kg and,
preferably from about 0.1 mg/kg to about 2 mg/kg. The
10 ability to produce large amounts of the recombinant
enzyme in accordance with this invention will permit
the evaluation of the therapeutic effect of
significantly larger doses.

15 5.3.2. IN VITRO USES OF α -Gal A

α -Gal A is a galactosyl hydrolase which has
activity toward various oligosaccharides,
glycoproteins, glycopeptides and glycolipids with
terminal α -galactosidic linkages. Thus, the enzyme
20 can be used in vitro to modify these α -galacto-
glycoconjugates. For example, the recombinant α -Gal A
of the invention could be utilized for a variety of
desirable modifications including but not limited to:
(a) the conversion of blood group B erythrocytes to
25 cells expressing the blood group O antigen (Harpaz, et
al., 1977, Eur. J. Biochem. 77:419-426); and (b) the
hydrolysis of stacchiose to raffinose, raffinose to
the disaccharide sucrose, or the hydrolysis of
melibiose to galactose and glucose (Silman, et al.,
30 1980, Biotechnol. Bioeng. 22:533). Such hydrolyses
have commercial applications as in the degradation of
molasses as a substrate for yeast production
(Liljestrom-Suominen, et al., 1988, Appl. Environ.
Micro. 54:245-249).

6. EXAMPLE: OVEREXPRESSION AND SPECIFIC
SECRETION OF BIOLOGICALLY ACTIVE
 α -GALACTOSIDASE A IN A MAMMALIAN CELL SYSTEM

The subsections below describe the production of
5 large quantities of human recombinant α -Gal A. A
full-length cDNA encoding human α -Gal A was inserted
into the expression vector p91023(B) in front of the
amplifiable dihydrofolate reductase (DHFR) cDNA. The
functional integrity of cDNA construct (p91-AGA) was
10 confirmed by transient expression of active enzyme in
COS-1 cells; 650 U/mg (nmol/hour) versus endogenous
levels of -150 U/mg of 4-MU- α -D-galactopyranoside
activity. The p91-AGA construct was introduced by
electroporation into DG44 dhfr⁻ CHO cells. Positive
15 selection in media lacking nucleosides resulted in the
isolation of clones expressing the active enzyme at
levels ranging from 300 to 2,000 U/mg. Selected
subclones, grown in increasing concentrations of
methotrexate (MTX, 0.02 to 1.3 μ M) to co-amplify DHFR
20 and α -Gal A cDNAs, expressed intracellular levels of
 α -Gal A activity ranging from 5,000 to 25,000 U/mg.
Notably, subclone DG44.5, which expressed high
intracellular levels of α -Gal A, secreted more than
80% of the total recombinant enzyme produced. At a
25 MTX concentration of 500 μ M, 10^7 cells secreted -15,000
U/ml culture media/day. Of note, endogenous CHO
lysosomal enzymes were not secreted including β -
hexosaminidase, α -mannosidase, β -galactosidase and β -
glucuronidase, indicating that the secretion was α -Gal
30 A specific and not due to saturation of the mannose-6-
phosphate receptor-mediated pathway. Using a hollow
fiber bioreactor, up to 5 mg per liter per day of
recombinant α -Gal A enzyme was produced per day. The
secreted α -Gal A was purified by affinity
35 chromatography for characterization of various
physical and kinetic properties. The recombinant α -

Gal A had a pI, electrophoretic mobility and Km values which were similar to the enzyme purified from human plasma. In addition, ³²P labeling studies revealed
5 that both the lysosomal and secreted forms were phosphorylated, presumably in their oligosaccharide moieties. Current studies are directed to characterize additional kinetic and physical
10 properties, the oligosaccharide moieties and the crystal structure of the recombinant enzyme. Furthermore, the availability of large amounts of soluble active enzyme will permit the evaluation of enzyme replacement in animal systems prior to clinical trials in hemizygotes with Fabry disease.

15

6.1. MATERIALS AND METHODS

6.1.1. MATERIALS

Restriction endonucleases, the Klenow fragment of
20 DNA polymerase I, T4 polymerase and T4 ligase were from New England Biolabs; α and γ -³²[P] dNTPs (3000 Ci/mole) and α ³⁵[S]dATP (100 Ci/mole) were from Amersham. The COS-1 cell line was purchased from ATCC, Rockville, MD. The CHO DG44 dhfr^r cell line is
25 described (Urlaug, et al., 1986, Somat. Cell Genet. 12:555-566).

6.1.2. CONSTRUCTION OF EXPRESSION VECTOR p91-AGA

30 Plasmid pcDAG126 (Bishop, et al., 1988, in, Lipid Storage Disorders, Salvaryre, R., Douste-Blazy, L. Gatt, S. Eds. Plenum Publishing Corporation, New York, pp. 809 to 822) containing the full-length α -Gal A cDNA was digested with Bam HI and Pst I and the 1.45
35 kb insert fragment was purified by agarose gel electrophoresis. The cDNA was then force-subcloned into plasmid pGEM-4 at the Bam HI and Pst I sites

resulting in pGEM-AGA126. This plasmid was then digested with Hind III, end-filled using Klenow and ligated to Eco RI linkers. After digestion with Eco RI, the 1.45 kb fragment was purified as above and cloned into the Eco RI site of the mammalian expression vector p91023(B) (Wong et al., 1985, Science 228:810) resulting in p91-AGA (FIG. 1G).

10 6.1.3. CELL CULTURE, ELECTROTRANSFECTION,
 AND GENE AMPLICATION

 COS-1 and DG44 CHO cells were maintained at 37°C in 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) and antibiotics; DG44 (dhfr⁻) cells were maintained by addition of 0.05 mM hypoxanthine and 0.008 mM thymidine to the media. Following transfection, the recombinant CHO lines were grown in DMEM supplemented with 10% dialyzed FCS in the absence or presence of MTX.

 For electroporation, cells were trypsinized and centrifuged at 800 x g at room temperature for 10 min. The pellet was washed once with DMEM supplemented with 10% FCS serum and twice in ice-cold electroporation buffer (phosphate buffered sucrose; 272 mM sucrose, 7 mM sodium phosphate, pH 7.4, containing 1 mM MgCl₂). Cells were then resuspended in phosphate buffered sucrose at -0.65 to 1.0×10^7 /ml. The cell suspension (0.8 ml) was placed in a 0.4 cm gap cuvette (Bio-Rad), 5-20 µg of plasmid DNA was added and kept on ice for 10 min. The cuvette was placed in the "Gene PulserTM" chamber (Bio-Rad) and pulsed once at 25 µF with 300 V for COS-1 cells or 400 V for CHO DG44 (dhfr⁻) cells, the optimized settings for the respective cell lines. The cuvette containing the pulsed cells was placed on ice for 10 min and then the cells were removed from

the cuvette and placed in 15 ml of DMEM supplemented with 10% FCS.

For transient expression, COS-1 cells were harvested at 72 hours and assayed immediately. For stable expression, the transfected DG44 cells were grown for 48 hours and then were removed from the culture dish by trypsinization and replated at a 1:15 ratio in DMEM supplemented with 10% dialyzed FCS. Media was replaced every four days. After two weeks of growth, cell foci became visible and individual clones were isolated with cloning rings. Clones which expressed the highest levels of α -Gal A were subjected to amplification en masse by step-wise growth in increasing concentrations of methotrexate (MTX), 0.02, 0.08, 1.3, 20, 40, 80, 250 and 500 μ M.

6.1.4. ENZYME AND PROTEIN ASSAYS

For enzyme assay, the cells in a 100 mm culture dish were washed twice with 5 ml of phosphate buffer saline (PBS) and scraped into a 12 ml conical tube using a rubber policeman. Following centrifugation at 800 x g for 10 min, the cells were resuspended in 1 ml of 25 mM NaPO₄ buffer, pH 6.0, and then disrupted in a Branson cup sonicator with three 15 second bursts at 70% output power. The sonicate was centrifuged at 10,000 x g for 15 min at 4°C and the supernatant was removed and assayed immediately. Alternatively, for rapid screening, cells were washed as above and 1 ml of lysis buffer (50 mM sodium phosphate buffer, pH 6.5, containing 150 mM NaCl, 1 mM EDTA, 1% NP-40, and 0.2 mM PMSF) was added to the dish. The lysed cells were incubated at 4°C for 30 min, the lysates collected and transferred to a 1.5 ml tube, centrifuged in a microfuge, and then the supernatant was removed for assay.

The α -Gal A activities in the cell lysates and media were determined using 5 mM 4-methylumbelliferyl- α -D--galactopyranoside (4MU- α -Gal) as previously described (Bishop, et al., 1980, In Enzyme Therapy in Genetic Diseases: 2. Desnick, R.J. (Ed.). Alan R. Liss, Inc. New York, p. 17). Briefly, a stock solution of 5 mM 4MU- α -Gal was prepared in 0.1M citrate/0.2M phosphate buffer, pH 4.6, in an ultrasonic bath. The reaction mixture, containing 10 to 50 μ l of cell extract and 150 μ l of the stock substrate solution, was incubated at 37°C for 10 to 30 min. The reaction was terminated with the addition of 2.3 ml of 0.1 M ethylenediamine. The fluorescence was determined using a Turner model 111 Fluorometer. One unit of activity is the amount of enzyme which hydrolyzes one nmol of substrate per hour. The activities of α -mannosidase, β -galactosidase, β -hexosaminidase, β -glucuronidase and acid phosphatase were determined using the appropriate 4-methylumbelliferyl substrate. Protein concentrations were determined by the fluorescamine method (Bohlen, et al., 1973, Arch. Biochem. Biophys. 155: 213) as modified by Bishop et al. (Bishop, et al., 1978, Biochim. Biophys. Acta 524: 109).

6.2. RESULTS

6.2.1. EXPRESSION OF HUMAN α -Gal A IN COS-1 CELLS

The full-length human α -Gal A cDNA was cloned into the expression vector p91023(B) (Wong, et al., 1985, Science 228: 810) and the construct, designated p91-AGA, was introduced into COS-1 cells by electroporation. Increased levels of α -Gal A activity were detected at 24, 48 and 72 hours after transfection (FIG. 2A), indicating the functional

integrity of the p91-AGA construct. At 72 hours after transfection, the α -Gal A activity increased about four-fold, while no increase in α -Gal A activity was observed in cells transfected with the p91023(B) vector containing the α -Gal A cDNA in the antisense orientation, nor in the cells that received no DNA. In addition, the β -galactosidase levels, determined as a lysosomal enzyme control, were not changed (FIG. 2B).

6.2.2. TRANSFECTION AND AMPLIFICATION OF α -Gal A IN DHFR CHO CELLS

Recombinant clones stably expressing human α -Gal A were obtained by electrotransfection of the p91-AGA construct into DG44 dhfr CHO cells and amplification of the integrated vector DNA with selection in increasing MTX concentrations. Initial growth in media lacking nucleosides resulted in the identification of over 100 clones expressing α -Gal A at levels ranging from 100 to 1,800 U/mg protein (Table I). Clones with the highest α -Gal A level were grown in the presence of 0.02 to 0.08 μ M MTX to amplify the integrated p91-AGA DNA. Table II shows that the intracellular α -Gal A levels in representative amplified clones increased 2 to 6 fold in 0.02 μ M MTX and up to 10 fold when further amplified in 0.08 μ M MTX.

TABLE I

5

Intracellular α -Galactosidase A Activity In
 DG 44 (dhfr⁻) CHO Cells* Following
 Electrotransfection with p91-AGA

10

CLONE	α -Gal A Activity (U/mg protein)
-------	--

Parental DG44:	497
----------------	-----

15

Transfected:

20

25

4	493
5	1,243
7	108
8	524
9	1,155
11	1,115
20	624
24	1,864
46	720
52	180

30

* Cells grown in DMEM supplemented with 10%
 dialyzed FCS.

35

TABLE II

5 Intracellular α -Galactosidase A Activities
 In p91-AGA Transfected DG44 (dhfr⁻) CHO Cells
 Following Initial Amplification In Methotrexate

10 CLONE α -Gal A
 (U/mg)

0.02 μ M MTX:

15 5 4,990
 9 2,900
 11 3,170
 46.1 1,230
 46.5 4,570
 46.12 4,100

20

0.08 μ M MTX:

25 5.3 23,400
 5.7 7,950
 5.9 14,680
 5.11 3,070
 9.1 10,290
 9.4 7,950
 9.6 3,860

30

6.2.3. HIGH LEVEL EXPRESSION CLONES
 SECRETE HUMAN α -Gal A

Among the positive clones amplified in the
 presence of 0.08 μ M MTX, clone 5.3 had the highest
 35 intracellular α -Gal A level (Table II) and therefore
 was chosen for further amplification. When grown in

the presence of 1.3 μ M MTX, the α -Gal A activity in the growth media of clone DG5.3 was determined to be 2,500 U/ml, or 25-fold greater than the level in untransfected parental DG44 cells (50 to 100 U/ml). Growth in the presence of increasing concentrations of MTX, resulted in increased intracellular and secreted α -Gal A activities (Table III). Interestingly, over 80% of the total α -Gal A produced was secreted and growth in increasing MTX concentrations continued to increase the percentage of enzyme secreted. Note that the data shown in Table III were obtained after the cells were amplified in the presence of the indicated MTX concentration and then assayed for α -Gal A activity after growth for three weeks in the absence of MTX, which accounts for their lower intracellular activities than during growth under selective pressure (Pallavicini, et al., 1990, Mol. Cell Biol. 10: 401; Kaufman, R.J., 1990, Meth. Enzymol, 185: 537; Kaufman, R.J., 1990, Meth. Enzymol, 185: 487).

25

30

35

TABLE III

5 Intracellular And Secreted α -Galactosidase A Activities In p91-AGA Transfected CHO Line DG5.3 Following Step-Wise Amplification In Methotrexate. Data Were Obtained On Clones After Three Weeks Of Growth In The Absence Of Methotrexate.

10 Methotrexate Concentration CHO Cells Media*

(μM) (U/mg) (U/ml)

15 Untransfected DG44: 250 100

Transfected p91AGA5-3:

0.00 375 150

0.02 550 265

20 0.08 600 560

1.3 2,560 2,090

20 6,270 6,530

40 5,795 6,855

80 6,365 8,750

25 250 5,720 9,945

500 12,560 18,140

* 10^7 cells and 10 ml of media for each Methotrexate concentration.

30

6.2.4. SPECIFIC SECRETION OF OVER-EXPRESSED LYSOSOMAL ENZYMES

To determine whether the secretion of α -Gal A was due to saturation of the receptors for lysosomal targeting, the culture media from clone DG5.3 was assayed for the presence of other lysosomal enzymes.

35

As shown in Table IV, the activities of seven representative lysosomal enzymes were not increased or were lower than those in the media of the DG44 parental cell line, indicating that the DG5.3 secretion of α -Gal A was specific.

To determine if the secretion was specific to clone DG5.3, another clone, DG9, which was not secreting α -Gal A (i.e., activity in media was 120 U/ml), was subjected to step-wise growth in increasing MTX concentrations (i.e., from 0.02 to 20 μ M MTX). After amplification in 20 μ M MTX, clone DG9 had intracellular and secreted levels of α -Gal A activity of 9,400 U/mg and 7,900 U/ml, respectively; i.e. 89% of the total α -Gal A activity produced was secreted.

TABLE IV

Lysosomal Enzyme Activities Secreted In Culture Media Of Transfected CHO Cells

20

Lysosomal Enzyme	CHO Cell Line	
	DG44 [*] Control	5-3 ₂₅₀ [*] α -Gal A
α -Galactosidase A	56	16,900
α -Arabinosidase	2.4	0.9
α -Fucosidase	341	358
β -Galactosidase	35.2	8.9
β -Gaucuronidase	90.0	53.7
β -Hexosaminidase	2,290	2,090
α -Mannosidase	147	82.8
Acid Phosphatase	30.6	6.1

35

* Average of Triplicate Determinations in Two Independent Experiments.

Since treatment of recombinant CHO cells with 50 mM butyrate has been shown to specifically increase transcription of the stably integrated p91023(B) vector in CHO cells (Dorner, et al., 1989, J. Biol. Chem. 264: 20602; Andrews & Adamson, 1987, Nucl. Acids Res. 15: 5461) another transfected clone, DG11, which was not amplified, was grown in the presence of 5 mM butyrate (Table V). The intracellular levels of α -Gal A activity increased from 259 U/mg to 687 U/mg. Notably, in the presence of butyrate, increased α -Gal A activity was secreted into the media (103 to 675 U/ml), suggesting that secretion occurred when the gene copy number increased (or, more precisely, the steady state of α -Gal A mRNA was increased). Incubation of butyrate-induced cells with 5 mM M-6-P (to prevent recapture of the secreted enzyme by the cell surface receptor) did not result a significant increase in the amount of α -Gal A secreted.

20

TABLE V

Butyrate Effect On α -Gal A Secretion in CHO DG11
 α -Gal A Activity

25

Clone	Cells	Media
	(U/mg)	(U/ml)
Control	259	102.6
30 Butyrate	687	675
Butyrate + 5 mM M-6-P	604	700

35

6.2.5. EFFECT OF SERUM CONCENTRATION ON SECRETION

To determine if the serum concentration of the growth media had an effect on the levels of

recombinant α -Gal A secretion, clone DG5.3 was grown in 100 mm culture dishes at a density of 5×10^6 cells per dish, in the presence of 0% to 10% dialyzed FCS for 5 days. There was no apparent effect on α -Gal A secretion in cells grown with 2.5% to 10% serum (FIG. 3A-3B). The decreased level of secretion by DG5.3 cells cultured in 0% and 1% serum presumably reflected the poor growth of these cells.

10

6.2.6. PRODUCTION IN BIOREACTORS

To produce large quantities of recombinant human α -Gal A, 10^8 cells of clone DG5.3 which had been grown in the presence of $500 \mu\text{M}$ MTX (DG5.3₅₀₀), were used to seed a hollow fiber bioreactor. As shown in FIG. 4, the level of α -Gal A produced increased to about 10,000 U/ml per day. This level remained constant for about three months. In addition, the serum concentration required by these cells in the bioreactor was step-wise decreased to 1% without seriously decreasing α -Gal A production (FIG. 4). A single 90-day run of this bioreactor resulted in >350 mg of active recombinant α -Gal A secreted into the culture media.

25

6.3. DISCUSSION

For human α -Gal A, post-translational modifications appear to be essential for stability and activity, as evidence by the fact that the unglycosylated enzyme expressed in E. coli was unstable and rapidly degraded (Hantzopoulos & Calhoun, 1987, Gene 57: 159). In addition, the α -Gal A subunit, which has four potential N-glycosylation sites, undergoes carbohydrate modification and phosphorylation for lysosomal delivery (Lemansky, et al., 1987, J. Biol. Chem. 262: 2062). Previous

35

-56-

characterization of α -Gal A purified from plasma and tissue identified their different carbohydrate compositions, the plasma glycoform having more sialic acid residues (Bishop, et al., 1978, *Biochim. Biophys. Acta* 524: 109; Bishop, et al., 1980, *Birth Defects* 16:1; p. 17; Bishop and Desnick, 1981, *J. Biol. Chem.* 256:1307). Moreover, clinical trials of enzyme therapy revealed that compared to the tissue-derived form, the plasma glycoform had a prolonged retention in the circulation and was more effective in depleting the circulating accumulated substrate following intravenous administration to patients with Fabry disease (Desnick, et al., 1979, *Proc. Natl. Acad. Sci. USA* 76: 5326). Thus, the amplified expression of human α -Gal A in CHO cells was chosen for the expression of this recombinant enzyme whose native composition includes galactosyl and sialic acid residues (Ledonne, et al., 1983, *Arch. Biochem. Biophys.* 224:186).

Although this is the first human lysosomal hydrolase to be successfully overexpressed, an unexpected finding was the secretion of over 80% of the enzyme produced. This could result from several different mechanisms including (a) saturation of the mannose-6-phosphate receptor pathways; (b) a mutation that alters a critical glycosylation site; (c) failure to expose the mannose-6-phosphate moiety for receptor binding; or (d) an unusually low affinity of recombinant α -Gal A for the mannose-6-phosphate receptor (Reitman & Kornfeld, 1981, *J. Biol. Chem.* 256: 11977; Lang, et al., 1984, *J. Biol. Chem.* 259: 14663; and, Gueze, et al., 1985, *J. Cell. Biol.* 101: 2253; for review see, Kornfeld & Mellman, 1989, *Ann. Rev. Cell. Biol.* 5: 483). If the secretion of α -Gal A was due to the saturation of the receptor-mediated

pathway, then it would be expected that the other endogenous lysosomal enzymes also would be secreted. However, the levels of secreted CHO hydrolases were unchanged, or decreased (Table IV). To rule out a possible mutation in the α -Gal A cDNA introduced during construction and integration of the vector (Calos, et al., 1983, Proc. Natl. Acad. Sci. USA 80: 3015), the integrated vector DNA was amplified by the polymerase chain reaction. Ten subclones were completely sequenced in both orientations, and no mutations were identified. In companion studies of the purified recombinant protein (described *infra*), it was shown that the mannose-6-phosphate moiety was present on the enzyme and that the enzyme bound efficiently to the immobilized mannose-6-phosphate receptor. Furthermore, to prove that the secretion of this protein in the expression system utilized was not α -Gal A dependent, the cDNA encoding another lysosomal hydrolase α -N-acetylgalactosaminidase, was inserted into p91023(B) and amplified in CHO cells. Analogous to the observations with α -Gal A, cells that were high expressors of α -N-acetylgalactosaminidase (α -GalNAc), also secreted the recombinant enzyme in the medium.

The presence of functional mannose-6-phosphate moieties on the secreted enzyme implied that perhaps a different mechanism was responsible for its secretion. In fact, many other secreted proteins have been shown to contain mannose-6-phosphate. Some of these proteins include lysosomal proteins while the location of others is not clear. These proteins include, proliferin (Lee & Nathans, 1988, J. Biol. Chem. 263: 3521) secreted by proliferating mouse placental cell lines; epidermal growth factor receptor in A-431 cells (Todderud & Carpenter, 1988, J. Biol. Chem. 263: 17893); transforming growth factor β 1 (Purchio, et

al., 1988, J. Biol. Chem. 263: 14211); uteroferrin, an iron containing acid phosphatase secreted in large amounts by the uterine endometrium of pigs (Baumbach, 5 et al., 1984, Proc. Natl. Acad. Sci. USA 81: 2985); and cathepsin L (MEP), a mouse lysosomal cysteine protease secreted by mouse NIH 3T3 fibroblasts (Sahagian & Gottesman, 1982, J. Biol. Chem. 257: 11145). Of interest, transformation of NIH 3T3 cells 10 with Kirstein virus results in a 25-fold increase in the synthesis of MEP causing this enzyme to be selectively secreted even though it contains functional mannose-6-phosphate moieties (Sahagian & Gottesman, 1982, J. Biol. Chem. 257: 11145).

15 Recently, the mechanism for the selective secretion of MEP has been identified and it involves an inherent low affinity of MEP for the mannose-6-phosphate receptor (Dong, et al., 1989, J. Biol. Chem. 264: 7377).

20 It is also notable that the plasma-directed overexpression of yeast vacuolar carboxypeptidase Y in yeast results in over 50% of the normally glycosylated protein secreted as the precursor form (Stevens, et al., 1986, J. Cell. Biol. 102: 1551). Similar 25 findings were observed for the yeast proteinase A gene (Rothman, et al., 1986, Proc. Natl. Acad. Sci. USA 83: 3248). Studies have suggested that the precursor glycoproteins have subcellular localization signals located within the N-terminal propeptide that are 30 recognized by the secretion pathway, thereby precluding delivery to the lysosome-like vacuole. It is notable that the secretion of these yeast genes is gene-dosage dependent and that a similar phenomenon is observed for the expression in CHO cells of human α - 35 Gal A. Also, it is of interest that the precursor form of the yeast enzymes was secreted. The plasma

form of α -Gal A is more sialyated and secreted, and others have shown that the lysosomal enzymes in human urine are the precursor forms (Oude-Elferink, et al., 1984, Eur. J. Biochem. 139: 489). However, N-terminal sequencing of recombinant α -Gal A expressed by DG44.5 revealed that the amino-terminus was identical to that of α -Gal A purified from human lung (Bishop, et al., 1986, Proc. Natl. Acad. USA 83:4859). Thus, it is possible that the high-level expression of human lysosomal hydrolases results in their secretion due to the inability to modify the precursor and/or inability of the subcellular localization machinery to accommodate the intracellular delivery of the overexpressed glycoprotein. However, this again would result in the secretion of other lysosomal enzymes. Since no other lysosomal enzymes are detected in the culture media, it is less likely that secretion of α -Gal A results from saturation of a component of the subcellular localization machinery.

Further studies, directed to determine amino acid, carbohydrate or other differences (e.g., sulfation) between the secreted and intracellular forms of recombinant α -Gal A may provide insights into the mechanism underlying the mislocalization and selective secretion of human α -Gal A. In addition, efforts to evaluate the generality of this observation should include the overexpression of other human lysosomal enzymes. The fact that large amounts of recombinant human α -Gal A are secreted by CHO cells permits the convenient production of the recombinant enzyme. Section 8, infra, describes a method for the purification of the recombinant enzyme and the characterization of its physical and kinetic properties including its receptor-mediated uptake by Fabry fibroblasts.

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7. EXAMPLE: PURIFICATION, CHARACTERIZATION AND PROCESSING OF RECOMBINANT α -Galactosidase A

The subsections below describe the purification of human α -galactosidase A cloned into the amplifiable eukaryotic expression vector, p91023(B), and overexpressed in Chinese hamster ovary (CHO) cells. The recombinant enzyme protein, was selectively secreted into the culture media and over 200 mg was purified to homogeneity by a Fast Protein Liquid Chromatographic procedure including affinity chromatography on α -galactosylamine-SepharoseTM. The purified secreted enzyme was a homodimeric glycoprotein with native and subunit molecular weights of about 110 and 57 kDa, respectively. The recombinant enzyme had a pI of 3.7, a pH optimum of 4.6, and a K_m of 1.9 mM toward 4-methylumbelliferyl- α -D--galactopyranoside. It rapidly hydrolyzed pyrene-dodecanoyl-sphingosyl-trihexoside, a fluorescently labeled analogue of the natural glycosphingolipid substrate, which was targeted with apolipoprotein E to the lysosomes of the enzyme-producing CHO cells. Pulse-chase studies indicated that the recombinant enzyme assumed its disulfide-defined secondary structure in <3 min, was in the Golgi by 5 min where it became Endo H resistant and was secreted into the media by 45-60 min. Both the intracellular and secreted forms were phosphorylated. The secreted enzyme subunit was slightly larger than the intracellular subunit. However, following endoglycosidase treatment, both subunits co-migrated on SDS-PAGE, indicating differences in the oligosaccharide moieties of the two forms. Treatment of the radiolabeled secreted enzyme with various endoglycosidases revealed the presence of three N-linked oligosaccharide chains, two high-mannose types (Endo H sensitive) and one complex type, the latter

being Endo H and F resistant. Analyses of the Endo H-released oligosaccharides revealed that one had two phosphate residues which specifically bound to immobilized mannose-6-phosphate receptors while the other was a hybrid structure containing sialic acid. These physical and kinetic properties and the presence of complex-type oligosaccharide chains on the recombinant secreted enzyme were similar to those of the native enzyme purified from human plasma. The secreted form of α -Gal A was taken up by cultured Fabry fibroblasts by a saturable process that was blocked in the presence of 2 mM mannose-6-phosphate indicating that binding and internalization were mediated by the mannose-6-phosphate receptor. The binding profiles of the recombinant secreted enzyme and the α -Gal A secreted by NH_4Cl -treated human fibroblasts to the immobilized receptor were identical. The production of large amounts of soluble, active recombinant α -Gal A in accordance with the invention, which is similar in structure to the native enzyme isolated from plasma, will permit further comparison to the native enzyme forms and the clinical evaluation of enzyme replacement in Fabry disease.

7.1. MATERIALS AND METHODS

7.1.1. MATERIALS

Endo- β -N-acetylglucosaminidase H (Endo H), endo- β -N-acetylglucosaminidase D (Endo D), endoglycosidase F (Endo F) and peptide:N-glycosidase F (PNGase F) were obtained from Boeringer Mannheim, Indianapolis, IN. [^{35}S]-methionine (>1,000 Ci/mmol), D-[2,6- ^3H]-mannose (60 Ci/mmol), ^{32}P -Phosphorus (10 mCi/ml) and Amplify were obtained from Amersham, Arlington Heights, IL.

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PansorbinTM was obtained from Calbiochem, San Diego, CA. 4-MU glycosides were obtained from Genzyme, Cambridge, MA. Freund's adjuvants, sphingomyelin (from brain) and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma, St. Louis, MO. QAE SephadexTM, SephadexTM G-25, octyl SepharoseTM and SuperoseTM 6 were obtained from Pharmacia-LKB, Piscataway, NJ. The TLC silica plates (cat. 5626) were purchased from EM Science, Gibbstown, NJ. The COS-1 cell line was obtained from the ATCC. All tissue culture reagents were obtained from Gibco, Grand Island, NY. Sinti Verse I scintillation cocktail was obtained from Fisher, Pittsburgh, PA. The immobilized mannose-6-phosphate receptor was obtained from Dr. Stuart Kornfeld, Washington University, St. Louis, MO. The pyrene-dodecanoyl-sphingosyl-trihexoside (P-C₁₂STH) was obtained from Dr. Shimon Gatt, Hebrew University, Israel. Apolipoprotein E was obtained from BTG Inc., Ness-Ziona, Israel.

7.1.2. CELL CULTURE

Cells were maintained at 37°C in 5% CO₂ in Dulbecco's Modification of Eagle's Medium (DMEM) with 10% fetal calf serum (FCS) and antibiotics. The DG44 line was cultured in DMEM supplemented with HT (hypoxanthine, thymidine, Sigma) while the recombinant CHO line DG5.3 received DMEM supplemented with 10% dialyzed FCS. (Kaufman, et al., 1988, J. Biol. Chem. 263: 6352).

7.1.3. PURIFICATION OF RECOMBINANT α -Gal A

Recombinant CHO culture media was collected (20 L) and concentrated to 500 ml using a PelliconTM cassette tangential-flow concentrator, with a molecular weight cutoff of 10,000 daltons (Millipore,

MA). The pH of the concentrate was adjusted to 4.7 to 5.0 with 10 N HCl and subsequently clarified by centrifugation at 10,000 x g in an RC-5 refrigerated centrifuge for 10 min.

All chromatographic steps were automated on an FPLC apparatus (Pharmacia) and were performed at room temperature. Approximately 100 ml of the media concentrate (-20 mg of α -Gal A enzyme protein) was applied to an α -Gal A affinity column (α -GalNH₂-SepharoseTM; 2.5 x 8 cm) (Bishop & Desnick, 1981, J. Biol. Chem. 256: 1307) pre-equilibrated with buffer A (0.1 M citrate-phosphate, pH 4.7, 0.15 M NaCl). The column was washed with buffer A until the protein concentration in the eluate returned to the pre-application level (-200 ml) and was eluted with 150 ml of buffer B (0.1 M citrate-phosphate, pH 6.0, 0.15 M NaCl, 70.4 mM galactose). The eluate was collected, concentrated to about 20 ml using an ultrafiltration cell, molecular weight cutoff 30,000 daltons, under positive nitrogen pressure (AmiconTM). The concentrate was mixed with an equal volume of buffer C (25 mM Bis-Tris, pH 6.0, 3 M (NH₄)₂SO₄), centrifuged at 10,000 x g and the pellet which contained up to 40% of the activity, was redissolved in buffer A and mixed with an equal volume of buffer C and centrifuged as above. The combined supernatants were applied to a column of Octyl-SepharoseTM (1.5 x 18 cm) pre-equilibrated with buffer D (25 mM Bis-Tris, pH 6.0, 1.5 M (NH₄)₂SO₄). The column was washed as above until the eluting protein concentration returned to pre-application levels (-100 ml) and the column was eluted with buffer E (5 mM sodium-phosphate, pH 6.0, 50% ethylene glycol). The product from three Octyl-SepharoseTM elutions, totalling approximately 75 ml, was concentrated as above to about 2 ml using an AmiconTM concentrator. The

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concentrate was finally applied to a column of Superose™ 6 (20-40 μm , Pharmacia, 1.6 x 100 cm) equilibrated in buffer F (25 mM sodium phosphate, pH 6.5, 0.1 M NaCl). The α -Gal A peak was collected, -20 ml, concentrated as above and stored in buffer F at 4°C.

7.1.4. ENZYME AND PROTEIN ASSAYS

α -Gal A was assayed using 4-methylumbelliferyl- α -D--galactopyranoside (4-MU- α -Gal) as previously described (Bishop, et al., 1980, In Enzyme Therapy in Genetic Diseases: 2. Desnick, R.J. (Ed.). Alan R. Liss, Inc. New York, p. 17). Briefly, a stock solution of 5 mM 4-MU was prepared in 0.1 M citrate-phosphate buffer, pH 4.6 solubilized in an ultrasonic bath. The reaction mixtures containing 10-50 μl of enzyme preparation or cell extracts and 150 μl substrate, were incubated at 37°C for 10-30 min. The reactions were terminated with the addition of 2.33 ml of 0.1 M ethylenediamine. One unit of activity is that amount of enzyme which hydrolyzes 1 nmol of substrate/hour.

Endo H, Endo D, Endo F and PNGase F digestions were performed as described (Tarentino, et al., 1989, Meth. Cell. Biol. 32: 111). Samples were diluted to 0.2-0.5% SDS before digestion. All reaction volumes were 50 μl . A drop of toluene was added to each reaction tube to prevent bacterial growth. Briefly, Endo H digestions (5 mU/reaction) were performed at 37°C overnight in 5 mM sodium citrate, pH 5.5 and 0.2 mM PMSF. Endo D digestions (10 mU/reaction) were performed at 37°C overnight in 0.2 M citrate phosphate buffer, pH 6.0 and 0.2 mM PMSF. Endo F digestions (50 mU/reaction) were performed overnight at 30°C in 0.17 M sodium acetate, pH 6.0, 1.6% NP-40 and 0.2 mM PMSF.

PNGase F digestions (100 mU/reaction) were carried out overnight at 30°C in 0.17 M potassium phosphate, pH 8.6, 1.6% NP-40, 0.2 mM PMSF.

5 Protein concentration was determined by the fluorescamine method (Bohlen, et al., 1973, Arch. Biochem. Biophys. 155: 213) as modified by Bishop et al. (Bishop et al., 1978, Biochim. Biophys. Acta 524: 109).

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7.1.5. IN VIVO NATURAL SUBSTRATE ASSAY

For this assay, 30 nmoles of P-C₁₂STH and 70 nmoles of sphingomyelin were mixed in a chloroform:methanol solution (1:1), evaporated under
15 nitrogen and dried in a Speed-VacTM (Savant). The pellet was resuspended in 2 ml of saline, sonicated using a Heat Systems Ultrasonics, Inc., Microson sonicator for 3-5 min at 40% output power and allowed to stand at room temperature for 1 hour.

20 Apolipoprotein E (80 µg) was added and the mixture was incubated for an additional 15 min at room temperature. The liposomes were added to the culture media of recombinant CHO cells and incubated at 37°C in a CO₂ incubator for 1 to 4 hours. Cells were
25 removed from the culture dishes by trypsinization, washed once in DMEM supplemented with 10% fetal calf serum and twice with saline. The cell pellet was resuspended in chloroform-methanol and heated to 60°C for 10 min and centrifuged at 600 x g for 10 min. The
30 supernatant was dried under nitrogen and the pellet resuspended in 100 µl of chloroform:methanol. Samples were spotted on a silica gel thin layer chromatography plate and chromatographed in chloroform:methanol:water (90:10:1) for 45 min followed by chromatography in
35 chloroform:methanol:water (75:25:4) for 30 min. Products were visualized under UV light (330 nm),

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excised from the plate by scraping, resuspended in chloroform:methanol, and their fluorescence quantitated in a FarrandTM spectrofluorometer (343 nm) 5 excitation, 378 nm emission).

7.1.6. POLYCLONAL ANTIBODIES

A New Zealand white rabbit (2 kg) was injected with 150 μ g of purified splenic α -Gal A in Freund's 10 complete adjuvant prepared as follows: 150 μ g of α -Gal A was added to 0.5 ml of PBS in a glass syringe. Using a stainless steel 21 guage needle, the PBS/ α -Gal A solution was mixed with 0.5 ml of Freund's complete adjuvant in a second glass syringe, until a homogenous 15 emulsion was obtained. The emulsion was injected into 8 different subcutaneous sites (back) and 1 intramuscular site (thigh). Two months following the initial injection, the rabbit was boosted with 50 μ g of α -Gal A in Freund's incomplete adjuvant as above. 20 Serum was collected from an ear vein at days 8 and 12 following the boost. The titer was checked using a standard ELISA assay (Johnstone & Thorpe, 1982, Immunochemistry in Practice. Balckwell Scientific Publications, Oxford). Subsequent boosts were given 25 approximately every two months followed by a bleeding 10 days later. A typical bleed yielded 30-40 ml of blood.

7.1.7. SDS-PAGE AND AUTORADIOGRAPHY

30 Polyacrylamide gel electrophoresis was carried out under reducing conditions (where appropriate) as described by Laemmli in a 1.5 mm thick slab containing 10% acrylamide (Laemmli, U.K., 1970, Nature 227: 680). The gel was fixed in 10% acetic acid and 20% methanol 35 for 30 min and then soaked in Amplify for 30 min with

agitation. Gels were vacuum dried for 90 min (Hoffer) and exposed to Kodak X Omat ARTM for 4 to 72 hours.

5 7.1.8. ISOELECTRIC POINT AND
 pH OPTIMUM DETERMINATION

The isoelectric point was determined using QAE sephadex essentially as described by Yang and Langer (Yang & Langer, 1987, Biotechniques 5: 1138). The pH optimum was determined in 25 mM sodium phosphate
10 buffer at 37°C.

 7.1.9. MANNOSE-6-PHOSPHATE RECEPTOR
 AFFINITY CHROMATOGRAPHY AND
 QAE SEPHADEXTM CHROMATOGRAPHY

15 The 215 kDa mannose-6-phosphate receptor (M-6-P receptor) coupled to Affigel-10 was at a concentration of 0.4 mg/ml of packed gel. Samples, in binding buffer (50 mM imidazole, pH 7.0, 150 mM NaCl, 0.05% TritonTM X-100, 5 mM sodium- β -glycerolphosphate, 0.02%
20 sodium azide), were applied to a 1.5 x 0.8 cm column at a flow rate of 0.3 ml/minute. Following sample application (5 ml), the column was washed with 5 ml of binding buffer and eluted with a nonlinear gradient of mannose-6-phosphate in binding buffer (0-5 mM). This
25 exponential gradient (Dong, et al., 1990, J. Biol. Chem. 265: 4210) was formed by an apparatus consisting of two chambers of 2.5 cm diameter and 1 cm diameter. Fractions were collected (0.5 ml) and 10 μ l aliquots assayed for α -Gal A activity using 4-MU- α -Gal, and for
30 radioactivity using 10 ml of Sinti Verse I scintillation cocktail.

 QAE SephadexTM chromatography in a 3 x 0.8 cm column was performed as described (Varki & Kornfeld, 1983, J. Biol. Chem. 258: 2808; Varki & Kornfeld,
35 1980, J. Biol. Chem. 255: 10847). Briefly, following digestion with Endo H, the released oligosaccharides

(labeled with [³H]-mannose) were isolated and desalted on an 18 x 0.8 cm column of Sephadex™ G-25. Samples were applied to the column of QAE Sephadex™ and eluted with successive 5 ml aliquots of 2 mM Tris, pH 8.0 containing 0, 20, 40, 80, 100, 120, 140, 160, 200, 400 and 1,000 mM NaCl. Oligosaccharides eluted according to the number of their negative charges; 0 charge at 0 mM NaCl, 1 at 20 mM NaCl, 2 at 70 mM NaCl, 3 at 100 mM NaCl and 4 at 140 mM NaCl.

7.1.10. LABELING OF CELLS WITH [³⁵S]-METHIONINE, [³H]-MANNOSE AND [³²P]-PHOSPHOROUS

Confluent cultures in 100 mm dishes were washed once with 5 ml of methionine-free DMEM. A fresh aliquot of this medium (5 ml) was placed in each dish and cultures were incubated in a 37°C incubator for 30 min. The media was removed from the dishes and a fresh aliquot of methionine-free DMEM (1 ml), supplemented with 10% dialyzed FCS and 50-100 µCi of [³⁵S]-methionine was added. Cells were incubated at 37°C for 3 to 5 min, the radioactive media was removed and cells washed twice with DMEM plus FCS. Cells were chased for the indicated times in 5 ml of DMEM plus FCS containing 2 mM methionine. For overnight labeling, cultures received 5 ml of methionine-free DMEM supplemented with dialyzed FCS, glutamine, antibiotics, 10 mM NH₄Cl and 200 µCi [³⁵S]-methionine.

For [³H]-mannose labeling, cultures were grown as above in supplemented DMEM. Cells were washed with 5 ml of low-glucose DMEM and a fresh aliquot of media was added. [³H]-mannose (250 µCi; dried under nitrogen and resuspended in DMEM), was added and cells were incubated in a 37°C incubator for 24 hours.

For ³²P labeling, cultures were switched to phosphate-free DMEM supplemented with 10% dialyzed

FCS. Following addition of [³²P]-orthophosphate (1 mCi) cultures were incubated in a 37°C, CO₂ incubator for 24 hours.

5

7.1.11. CELL LYSIS AND IMMUNOPRECIPITATION

Cells grown in 100 mm culture dishes were washed twice with 5 ml of phosphate buffered saline (PBS) and scraped into 12 ml conical tubes using a rubber policeman and 10 ml of PBS. Following centrifugation at 2,500 rpm for 10 min cells were resuspended in 1 ml of 25 mM NaPO₄, pH 6.0 and received three 15-second bursts in a Branson cup sonicator. Cell debris was removed by centrifugation (10,000 x g for 15 min at 4°C). Alternatively, cells were washed as above and 1 ml of lysis buffer (50 mM sodium phosphate, pH 6.5, 150 mM NaCl, 1mM EDTA, 1% NP-40, 0.2 mM PMSF) was added to the dish. The culture dish was incubated at 4°C for 30 min and cells were transferred to a 1.5 ml microcentrifuge tube. Cell debris was removed as above.

Immunoprecipitation was carried out as described (Sambrook, et al., 1989, Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory Press pp. 18.42-18.46). Briefly, 0.5 ml of cell lysate or culture media was placed in a 1.5 ml microcentrifuge tube and 50 µl of preimmune rabbit serum was added. The mixture was incubated at 4°C for 1 hour with gentle agitation. Fifty µl of PansorbinTM was added and incubation was continued for 30 min. The mixture was clarified by centrifugation at 10,000 x g for 5 min, 100 µl of anti-α-Gal A polyclonal antibody was added and incubation was continued for 1 hour at 4°C with gentle rocking. Pansorbin (100 µl) was added and incubation continued for 30 min as above. The tertiary S. aureus cells-antibody-antigen complex was

collected by centrifugation as above. The supernatant was discarded and the pellet washed successively in NET buffer (50 mM sodium phosphate, pH 6.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% gelatin) supplemented with 0.5 M NaCl, in NET buffer with 0.1% SDS and in TN buffer (10 mM Tris, pH 7.5, 0.1% NP-40). The immunoprecipitated protein was denatured by heating at 100°C for 5 min in the presence of 2% SDS, 100 mM DTT (DTT was not used for experiments involving secondary structure conformations). S. aureus cells were removed by centrifugation at 10,000 x g for 5 min at room temperature.

7.2. RESULTS

7.2.1. PURIFICATION

Recombinant α -Gal A produced in the cell bioreactor was purified from the crude media by affinity chromatography on α -GalNH₂-C₁₂-Sepharose™ (Bishop & Desnick, 1981, J. Biol. Chem. 256: 1307) followed by hydrophobic chromatography on Octyl Sepharose™ and gel filtration on a 100 cm Superose™ 6 column as described above. Table VI shows a typical purification of a 20 mg lot of recombinant α -Gal A and the specific activities of the enzyme at each stage of purification. The recombinant enzyme was essentially homogeneous, following the gel filtration step, and was >98% pure as judged by SDS-PAGE (FIG. 5). A minor contaminant of bovine serum albumin was removed by an additional gel filtration step on a column of Blue-Sepharose (Travis, et al., 1976, Biochem. J. 157: 301) resulting in an enzyme preparation which was greater than 99% pure as judged by loading 20 μ g of α -Gal A on SDS-PAGE.

TABLE VI

FPLC Purification Of Recombinant α -GAL A
 A Typical Purification Run Starting
 With 20 MG Of α -GAL A

Step	U x 10 ³	U x 10 ³	Fold Purification	Yield %
Media	39,750	5	1	100
α -GalNH ₂ -Sephacrose TM	36,500	680	136	91
Octyl Sephacrose TM	31,750	3,400	680	79
Superose TM 6	30,800	4,150	830	78

That the purification of recombinant α -Gal A would be facilitated by growth of the CHO cells in serum-free media was demonstrated by metabolic labelling of total cellular and secreted protein. In contrast to the result seen in FIG. 5, radiolabeled α -Gal A was essentially the only protein seen in the media of the high-expressor line, DG5.3 (FIG. 6).

7.2.2. PHYSICOKINETIC PROPERTIES

Recombinant α -Gal A was found to have a subunit molecular weight of 57 Kd based on SDS-PAGE (FIG. 5). The Km towards the artificial substrate 4-MU- α -D--galactopyranoside was 1.9 mM (FIG. 7A) and the pH optimum and isoelectric point were 4.6 and 3.7 respectively (FIG. 7B and 7C).

In order to determine whether the recombinant enzyme recognized and hydrolyzed its natural substrate, liposomes containing the fluorescently-labeled α -Gal A substrate P-C₁₂STH and apolipoprotein E

(for lysosomal targeting) were incubated with CHO cells over-expressing α -Gal A (clone DG5.3). As shown in FIG. 8A-8B, recombinant lysosomal α -Gal A rapidly hydrolyzed the substrate to P-C₁₂SDH (the dihexoside). The rapid hydrolysis of P-C₁₂STH indicates that recombinant α -Gal A can recognize this natural substrate analog and very efficiently hydrolyze it. Also, since this substrate is targeted to the lysosome, cell associated recombinant α -Gal A must be correctly targeted to this location. These results indicate that recombinant α -Gal A produced and secreted by CHO cells is essentially identical to the enzyme purified from human plasma (Table VII).

15

TABLE VII

Property Comparison Of Recombinant α -GALACTOSIDASE A Enzyme Purified From Human Tissue

20

Property	α -Gal A		
	Spleen	Plasma	Recombinant
MW-Subunit, (KDa)	53	57	57
pH Optimum	4.5	4.6	4.6
Isoelectric Point, PI	4.3	3.7	3.7
Km (4-MU- α -D-Gal), mM	2.5	1.9	1.9
Phosphorylation (M-6-P)	+	?	+
Natural Substrate Hydrolysis (GL-3)	+	+	+

35

7.2.3. PROCESSING AND RATE OF SECRETION
OF RECOMBINANT α -Gal A

Nascent polypeptides, transversing the
5 endoplasmic reticulum assume secondary structure
conformations cotranslationally or soon after their
synthesis is completed (Gething, et al., 1989, Meth.
Cell. Biol. 32: 185). α -Gal A was labeled with [³⁵S]-
methionine for three min and then chased with cold
10 methionine for the indicated times.

Immunoprecipitated α -Gal A was visualized on SDS-PAGE.
The samples were prepared without DTT in order to
maintain disulfide bridges that might have formed
during the chase, indicative of a secondary structure
15 conformation. A control (+DTT) was prepared by boiling
an aliquot of the 60 minute sample in the presence of
DTT to destroy disulfide bonds and the secondary
structure. At 0 min of chase (after 3 min of
labeling) there was already a change in the mobility
20 of this enzyme indicating that conformational changes
occur cotranslationally or soon after completion of
synthesis (FIG. 9).

Arrival of the new polypeptide to the Golgi
network was detected by the acquisition of Endo H
25 resistant oligosaccharides (Gething, et al., 1989,
Meth. Cell. Biol. 32: 185). Radiolabeled α -Gal A (3
minute pulse) was chased with nonradioactive
methionine and immunoprecipitated as above. The
immunoprecipitates were then treated with Endo H and
30 visualized on SDS-PAGE. Between 2 and 7 min of chase,
the first Endo H-resistant form of α -Gal A could be
detected, indicative of arrival of the recombinant
enzyme at the Golgi, about 5 to 10 min following its
synthesis (FIG. 10). The majority of the Endo H
35 sensitive form was rendered resistant by 60 min of
chase.

This enzyme transverses the Golgi network and is secreted at 45 to 60 min of chase (FIG. 11). Analysis of total media, from [³⁵S]-methionine labeled cells, revealed that >95% of the secreted protein by the recombinant CHO cells was α -Gal A (FIG. 12).

7.2.4. ANALYSIS OF CARBOHYDRATE MOIETIES ON RECOMBINANT α -Gal A

There are four N-glycosylation consensus sequences (Asn-X-Ser/Thr) in the α -Gal subunit predicted by the cDNA sequence. The fourth site is probably not utilized since it contains a proline residue in the X position. Recombinant α -Gal A was digested with Endo H, Endo F, Endo D and PNGase F. Digestion with PNGase F caused an [~]7 kDa shift in mobility on SDS-PAGE of half of the α -Gal A (FIG. 13). This change in molecular weight can be attributed to the removal of 3 N-linked carbohydrate moieties. Digestion of the recombinant enzyme with a cocktail of Endo H, Endo F and PNGase F did not result in any further decrease in molecular weight, indicating that all of the enzyme contains three N-linked carbohydrate moieties.

Endo D, a glycosidase with a strict specificity for the lower Man α 1-3 branch of the high-mannose core pentasaccharide (Tarentino, et al., 1989, Meth. Cell. Biol. 32: 111), did not have an effect on the mobility of α -Gal A, indicating that the recombinant enzyme does not contain this type of oligosaccharide (FIG. 13). Endo H and Endo F together resulted in a 4 kDa shift indicating that two out of the three oligosaccharides on this enzyme are of the high-mannose type (Varki & Kornfeld, 1980, J. Biol. Chem. 225: 10847).

Interestingly, intracellular α -Gal A was completely sensitive to PNGase F while half of the

-75-

secreted enzyme was partially resistant to PNGase F (FIG. 14). Since this resistance was eliminated by co-treatment with Endo H and Endo F (FIG. 13), further studies are necessary with Endo H and Endo F separately to determine the molecular nature of either the selective inhibition of PNGase F or the resistance of a proportion of the recombinant secreted enzyme to PNGase F digestion.

10 Having determined that the recombinant enzyme contains three oligosaccharides, two of which are of the high-mannose type, the effect of inhibition of glycosylation was investigated (Furhmann, et al., 1985, Biochim. Biophys. Acta 825: 95). Processing and secretion of recombinant α -Gal A is not affected by selective inhibition of oligosaccharide processing. In the presence of deoxynojirimycin (an inhibitor of glucosidase I and II), deoxymannojirimycin (an inhibitor of mannosidase I), and swainsonine (an inhibitor of mannosidase II) α -Gal A secretion rate remains the same as the controls (FIG. 15). However, tunicamycin (an inhibitor of oligosaccharide addition) inhibits secretion of α -Gal A by as much as 80% (FIG. 15). The secreted enzyme from tunicamycin-treated cultures could bind to a Con A SepharoseTM column indicating that this enzyme is partially glycosylated, probably due to incomplete inhibition of glycosylation by tunicamycin. These results indicate that oligosaccharide addition but not the processing events tested is necessary for maturation and secretion of the recombinant enzyme.

7.2.5. PHOSPORYLATION

Since the recombinant α -Gal A contained high-mannose moieties, the recombinant enzyme could contain M-6-P and be competent for receptor mediated uptake.

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Cells from clone DG5.3 were metabolically labeled with [³²P]-orthophosphate for 12 hours and then the cell extracts and media immunoprecipitated and visualized on SDS-PAGE. As shown in FIG. 16, both cell-associated and secreted α -Gal A were phosphorylated, presumably at their carbohydrate moieties as suggested by the in vitro experiments described above.

10 7.2.6. ANALYSIS OF ENDO H SENSITIVE OLIGOSACCHARIDES

The high-mannose oligosaccharides were removed by treating immunoprecipitated [³H]-mannose labeled α -Gal A with Endo H. These oligosaccharides were analysed by chromatography on QAE SephadexTM (Varki & Kornfeld, 15 1983, J. Biol. Chem. 258: 2808; and, Varki & Kornfeld, 1980, J. Biol. Chem. 255: 10847). Two major forms of these oligosaccharides were detected, a form with 2 negative charges and one with 4 negative charges (FIG. 17A). The negative charge can be contributed by a phosphodiester moiety (-1), a phosphomonoester moiety (-2) or sialic acid (-1). Treatment of these sugars with dilute HCl did not shift the profile of any of the peaks indicating that there are no phosphodiester groups on these sugars (FIG. 17B) (Varki & Kornfeld, 25 1983, J. Biol. Chem. 258: 2808). Treatment with neuraminidase causes a shift of the -2 peak resulting in two new peaks at 0 and -1 negative charges (FIG. 17C). Therefore, the charge of the -2 peak is contributed by sialic acid, most likely two moieties. 30 The resulting -1 peak following neuraminidase treatment is probably a partial digestion of the -2 peak by the enzyme. Treatment of these oligosaccharides with alkaline phosphatase caused a shift of the -4 peak to 0 negative charge (FIG. 17D). 35 There was no effect on the -2 peak, indicating that the charge of the -4 peak is contributed by two

phosphomonoester bonds while the -2 peak does not contain any such bonds. Thus, it is evident from these results that Endo H releases two types of high-mannose oligosaccharides from recombinant α -Gal A, one containing sialic acid (possibly a hybrid oligosaccharide) and the other containing 2 phosphomonoester bonds (presumably as mannose-6-phosphate).

10 To further confirm these findings peaks -2 and -4 were chromatographed on an immobilized mannose-6-phosphate receptor column (FIG. 18). Although peak -4 interacted weakly with the receptor, it could be bound to the column and required the addition of mannose-6-phosphate for elution. A very weak interaction was 15 observed between the receptor column and the -2 peak, suggesting that a portion of these hybrid oligosaccharides may contain M-6-P.

The weak interaction of the high-mannose 20 oligosaccharides with the M-6-P receptor could be explained by the absence of the protein core (Varki & Kornfeld, 1983, J. Biol. Chem. 258: 2808). DG5.3 cells were labeled with [³⁵S]-methionine and the secretions chromatographed on a column of immobilized 25 mannose-6-phosphate receptor. Notably, the recombinant enzyme bound strongly to the column was eluted specifically by the addition of 5 mM M-6-P (FIG. 19).

30 7.2.7. INTERACTION OF α -Gal A WITH THE MANNOSE-6-PHOSPHATE RECEPTOR

Since recombinant α -Gal A has been shown to contain mannose-6-phosphate moieties, it was important to establish whether this was also true for normal 35 human α -Gal A. CHO proteins were labeled with [³⁵S]-methionine in the presence of NH₄Cl, to cause quantitative secretion of newly synthesized lysosomal

enzymes (Dean, et al., 1984, Biochem. J. 217: 27).
The media was collected and chromatographed on a
column of immobilized mannose-6-phosphate receptor.
5 The column was eluted with a gradient of mannose-6-
phosphate as described above. This elution protocol
can separate lysosomal enzymes into low and high
affinity receptor-binding ligands (Dong & Sahagian,
1990, J. Biol. Chem. 265: 4210).

10 The recombinant enzyme co-eluted with the bulk of
the lysosomal enzymes at an M-6-P concentration
indicative of high affinity forms (FIG. 20A). The
same experiment was performed with secretions of MS914
(normal diploid human fibroblasts) cells (FIG. 20B)
15 and 293 cells (human adenovirus transformed embryonic
kidney cells) (FIG. 20C). When the same M-6-P
gradient was applied, human α -Gal A also co-eluted
with the bulk of the lysosomal enzymes, demonstrating
that the recombinant enzyme exhibits affinity to the
20 M-6-P receptor similar to that of the normal human
enzyme.

7.2.8. RECEPTOR MEDIATED UPTAKE OF RECOMBINANT α -Gal A IN FABRY FIBROBLASTS

25 Fabry fibroblasts were incubated with varying
amounts of the recombinant enzyme for 6 hours (FIG.
21). The enzyme uptake was saturatable and was
specifically inhibited by the addition of 2 mM M-6-P
in the uptake media, indicating that the uptake was
via the cell surface M-6-P receptor.
30

8. EXAMPLE: α -Gal A-PROTEIN A FUSION EXPRESSED IN MAMMALIAN CELLS

The subsections below describe a fusion construct
of the human α -Galactosidase A cDNA and the
35 staphylococcal protein A IgG binding domain E
expressed in COS-1 cells and purified to apparent

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homogeneity by IgG affinity chromatography. The fusion construct was engineered using PCR techniques to insert the 16 nucleotide collagenase cleavage recognition sequence between the α -Gal A and the protein A domain E sequence. In addition, the termination codon was deleted from the α -Gal cDNA and inserted at the terminus of the domain E sequence. Transient expression of the fusion construct in COS-1 cells resulted in a 6 to 7-fold increase over endogenous levels of α -Gal A activity and significant secretion into the media (4,000 units; nmoles/hour). The fusion protein from the culture media was purified to homogeneity on IgG sepharose chromatography. After collagenase treatment, the liberated α -Gal A was separated from the protein A peptide by IgG chromatography. By this method over 85% of secreted α -Gal A fusion protein was purified as the active, glycosylated homodimeric protein. This method should be useful for the expression and rapid purification of normal and mutant proteins. In addition, this construct has been inserted into the CHO DG44 cells so that large amounts of the secreted recombinant enzyme can be produced and rapidly and efficiently purified.

25

8.1. MATERIALS AND METHODS

8.1.1. MATERIALS

Restriction endonucleases, Taq polymerase, T4 ligase and pGem plasmids were obtained from Promega (Madison, WI). Vector pRIT5 and IgG-Sepharose were purchased from Pharmacia (Piscataway, NJ). Sequenase sequencing kits were from United States Biochemical Corp. (Cleveland, OH). Collagenase was obtained from Sigma (St. Louis, MO). Oligonucleotides were

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-80-

synthesized using an Applied Biosystems DNA synthesizer model 380B.

5 8.1.2. CELL CULTURE AND TRANSFECTIONS

COS-1 cells were obtained from the ATCC (Rockville, MD). The cells were cultured by standard techniques in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal calf serum and antibiotics.

10 Exponentially growing COS-1 cells (5×10^6 cells /T75 flask) were detached from the plastic by trypsinization, collected by centrifugation at 3,000 x g, and then washed once in ice-cold electroporation buffer (phosphate buffered sucrose: 272 mM sucrose, 7
15 mM sodium phosphate, pH 7.4, containing 1 mM $MgCl_2$). Following centrifugation at 3,000 x g, the cells were resuspended in 0.8 ml of electroporation buffer and placed in an electroporation cuvette with a 0.4 cm gap. Ten to fifteen μg of plasmid DNA was added and
20 cells were kept on ice for 5 min. The cell-containing cuvette was placed in a Gene PulserTM electroporation apparatus (Bio-Rad) and the cells were pulsed at 350 V, 25 μF . The cells were maintained on ice for an additional 10 min and then placed into a 100 mm
25 culture dish containing 10 ml of growth medium.

8.1.3. PCR, DNA SEQUENCING AND VECTOR CONSTRUCTIONS

The fusion construct was synthesized using a recently described PCR technique (Ho, et al., 1989, Gene 77: 51; Kadowaki, et al., 1989, Gene 76: 161).
30 Briefly, the full-length α -Gal A cDNA was subcloned into the pGEM plasmid and the resulting pG6-AGA plasmid was used for PCR amplification of the α -Gal A sequence with primers designed to delete the
35 termination codon, to add a collagenase cleavage consensus sequence at the 3' end and to include an Eco

RI recognition sequence at the 5' end of the cDNA (FIG. 22). The sense primer was 5'-CCGAATTCATGCTGTCCGGTCACCGTG-3' and the antisense primer was 5'-CGCCGGACCAGCCGGAAGTAAGTCTTTTAATG-3'. The protein A domain E (Nilsson, et al., 1985, EMBO J. 4: 1075) was similarly amplified with the collagenase consensus sequence in the 5' oligonucleotide; the sense and antisense oligonucleotides were 5'-CCGGCTGGTCCGGCGCAACACGATGAAGCT-3' and 5'-GGCCGAATTCCGGGATCCTTATTTTGGAGCTTGAGA-3', respectively. The 1323 nt and 201 nt products of the α -Gal A and protein A PCR reactions were gel-purified on an 0.8% agarose gel and mixed together for the fusion PCR reaction. The sense primer from the α -Gal A reaction and the antisense primer from the protein A reaction were used for the final fusion reaction. The product of this reaction was digested with Eco RI and ligated into the Eco RI digested plasmid pGEM4Z. The protein A domain E and junctions between the linker and α -Gal A and protein A were confirmed by the dideoxynucleotide chain termination sequencing method of Sanger (Hanahan & Meselson, 1985, Methods Enzymol. 100: 333). The confirmed fusion sequence then was digested with Eco RI and subcloned into the eukaryotic expression vector p91023(B).

8.2. RESULTS

8.2.1. CONSTRUCTION OF α -Gal A-PROTEIN A (AGA-PA) FUSION

FIGURE 22 shows the strategy used for the construction of the α -Gal A-Protein A domain E fusion sequence. The full-length α -Gal A cDNA (1323 nt) and protein A domain E sequence (201 nt) were amplified separately and then fused by a second PCR amplification (FIG. 22) using the 5' α -Gal A cDNA

sense primer (P1) and the 3' Protein A antisense primer (P4). The primers were designed to (a) eliminate the α -Gal A TAA stop codon; (b) insert the
 5 16 nt collagenase cleavage consensus recognition sequence encoding Pro Ala Gly Pro between the α -Gal A and protein A cDNA sequence; and (c) introduce a TAA stop codon at the 3' end of protein A domain E. The integrity of this construct was confirmed by
 10 sequencing the protein A domain, linker and 3' of the α -Gal A cDNA (FIG. 23).

8.2.2. EXPRESSION OF pAGA-PA IN COS-1 CELLS

Seventy-two hours after transfection with the
 15 pAGA-PA construct, maximal levels of 4MU α -Gal activity were detected in cell extracts and in the spent culture media (Table VIII).

TABLE VIII

Transient Expression Of AGA-PA Construct In COS-1 Cells. Following Transfection A 7-Fold Increase In Endogenous α -Gal A Activity Was Observed. Also, An Increase Of α -Gal A In The Culture Media Was Observed

COS-1 CELLS	α -Gal A Activity*	
	Cells (U/mg)	Media (U/ml)
Control	210	0
Transfected	1,300	400

*Assayed using 4MU- α -Gal as substrate.

Compared to the endogenous α -Gal A activity in COS-1 cells of 210 U/mg, the transfected cells expressed 1300 U/mg. No α -Gal A activity was detected in the spent culture medium of uninfected COS-1 cells whereas 72 hours after transfection, 400 units of activity were secreted into the media.

8.2.3. AFFINITY PURIFICATION OF α -Gal A

The spent media from a single 100 mm dish of COS-1 cells was collected 72 hours after transfection and applied to a column of IgG-SepharoseTM. Minimal activity of α -Gal A passed through the column during sample application (flow-through), or during the buffer wash (Table IX). However, more than 95% of the bound α -Gal A fusion protein was eluted by the addition of 0.5 M acetic acid (elution buffer).

20

TABLE IX

IgG SepharoseTM Chromatography Of The α -Gal A Protein A Fusion Product From The Culture Media of Transfected COS-1 Cells

<u>Purification Step</u>	<u>α-Gal A Activity* (U/ml)</u>
Medium	4,400
Flow-Through	10
Buffer Wash	0
Elution**	4,200

30

* Ten ml of culture media were applied to the column, washed and eluted as described in "Methods". α -Gal A activity was assayed using 4MU- α -Gal as substrate.

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** 0.5 M HAc, pH 3.4

8.2.4. RELEASE OF THE PROTEIN A DOMAIN
FROM THE AGA-PA FUSION PROTEIN

The affinity purified fusion protein was treated with collagenase for 1 hour and the reaction products were rechromatographed on the IgG affinity column (Table X). The Protein A domain E was readily bound to the IgG column, whereas the human α -Gal A was eluted in the flow-through. Almost 90% of the applied activity was eluted. Based on the specific activity of the purified enzyme, it was estimated that this procedure resulted in 90% pure enzyme.

TABLE X

Treatment Of α -Gal A-Protein Fusion With Collagenase. Upon Treatment The Binding Of The IgG Column Decreased From 69% To 11%.

% Of Recovered α -Gal A Activity*

STEP	COLLAGENASE**	
	-	+
Flow-Through	31	89
Elution	69	11

* Assayed using 4MU- α -Gal as substrate; a total of 4,200 units of α -Gal A activity was applied.

** Collagenase treatment for 1 hour at 25°C.

9. EXAMPLE: IN VIVO MODIFICATION OF
RECOMBINANT HUMAN α -Gal A GLYCOSYLATION
BY α 2, 6-SIALYLTRANSFERASE

The example presented here describes a method
5 whereby recombinant human α -Gal A enzyme is produced
in a form that more closely resembles the native
plasma glycoform of the enzyme. Specifically, human
 α -Gal A is produced in CHO cell lines that have been
engineered to contain an α -2,6-sialyltransferase gene
10 such that the α -Gal A protein made in these cell lines
is sialylated. Results presented below demonstrate
that the biologically active α -Gal A enzyme produced
using such cell lines exhibit a broader tissue
distribution, which can enhance this recombinant α -Gal
15 A's therapeutic value.

9.1. MATERIALS AND METHODS

20 9.1.1. CONSTRUCTION OF THE α 2,6-
SIALYLTRANSFERASE EXPRESSION
VECTOR, pST26

The 1.6 kb rat CDNA (ST3) encoding the complete
amino acid sequence of the β -galactoside α 2,6-sialyl-
transferase (Gal α 2,6ST; Weinstein et al., 1987, J.
25 Biol. Chem. 262: 17735) was subcloned into the Bam HI
site of the mammalian expression vector pRLDN, a gift
from Smith, Kline and French Laboratories, resulting
in the vector designated pST26. This construct was
introduced by electroporation into the CHO cell line
30 DG5.3-1000Mx, a high overexpressor of human α -
galactosidase A. Clones were selected by growth in
media containing 500 μ g/ml of G418 to select for
expression of the pST26 neo gene. Positive clones
were individually isolated using cloning rings and
35 then each was analyzed for expression of total
secreted recombinant human α -galactosidase A. In
addition, the percent of the secreted recombinant α -

galactosidase A that contained α 2,6-sialic acid residues was determined by Sambucus nigra agglutinin (SNA) chromatography on a column of SNA-SepharoseTM as described by Lee et al. (1989, J. Biol. Chem. 264:13848-13855).

9.1.2. SNA-LECTIN FLUORESCENCE MICROSCOPY

For fluorescence microscopy, positive DG5.3-1000Mx-ST26 clones were grown on 12-mm glass coverslips and then stained with fluorescein isothiocyanate (FITC)-tagged SNA as previously described (Lee et al., 1989, J. Biol. Chem. 264:13848-13855). Briefly, the cells were fixed with a fresh solution of 2% para-formaldehyde, 0.1% glutaraldehyde in phosphate buffered saline (PBS), pH 7.0, for 1 hr at 23°C, and subsequently blocked with 50 mM NH₄Cl in PBS for 30 min at 23°C. Cells were incubated with FITC-SNA at a concentration of 25 mg/ml in PBS for 30 min at 23°C. Following washing in PBS, the coverslips were mounted in 15% vinol 205 polyvinol alcohol, 33% glycerol, 0.1% sodium azide in 100 mM Tris, pH 8.5. The cells were viewed with a Zeiss Photomat III fluorescence microscope and were photographed using Kodak GoldTM color film, ASA 25.

9.1.3. PURIFICATION OF RADIOLABELLED HUMAN α -GAL A WITH AND WITHOUT α 2,6-SIALIC ACID RESIDUES

Parental DG5.3-1000Mx cells and modified DG5.3-ST26.1 cells were grown in 1 liter roller bottles ($\sim 5 \times 10^8$ cells) with 125 ml of DMEM, containing 10% dialyzed fetal calf serum (GIBCO, Grand Island, NY) and 500 μ Ci [³⁵S]-methionine. The radioactive medium was replaced daily for three days, and the spent medium from each line was collected, pooled and concentrated using a stirred cell concentrator

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(Amicon, Beverly, MA). Approximately 2 mg (4×10^6 U) of recombinant enzyme secreted from the DG5.3-1000Mx and DG5.31000Mx-ST26.1 cell lines, respectively, was individually purified to a specific activity of 100 cpm/U of enzyme. The purified, radiolabeled, secreted recombinant α -galactosidase A preparations (0.5 mg each) were treated individually with 5 units of acid phosphatase (Sigma) and the dephosphorylated forms also were used for intravenous injections.

9.1.4. IN VIVO HALF-LIFE AND TISSUE DISTRIBUTION OF RECOMBINANT α -GAL A

For the plasma half-life determinations, each radiolabelled α -Gal A form was injected into the tail vein of two CDI female mice. Each animal was bled through the sinus plexus at timed intervals over a 10-20 min period. Blood samples were centrifuged in an IEC Microhematocrit centrifuge and 50 μ l of plasma was added to an aqueous scintillation fluid (AquaSolTM, NEN, Boston, MA) for counting. To assess the tissue distribution of the different enzyme forms, two mice for each enzyme were sacrificed by decapitation 4 hr after enzyme injection, selected tissues were removed, and the radioactivity in various tissues was determined and expressed per gram of tissue wet weight. Each value is the average of two independent experiments.

9.2. RESULTS

9.2.1. INTRODUCTION OF PST26 INTO DG5.3-1000Mx CHO CELLS AND DEMONSTRATION OF α 2,6-SIALYLTRANSFERASE ACTIVITY IN G418-SELECTED CLONES

Following electroporation of the pST26 plasmid into CHO DG5.3-1000MX cells, transformed cells were selected which were resistant to the antibiotic G418.

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From the pool of resistant cells, 10 individual clones were isolated and expanded for further studies. These clones were designated DG5.3-1000Mx-ST26.1 to ST26.10. As evidence for the presence of α 2,6-sialyltransferase activity in these cells, each cell line was stained with FITC-SNA and then examined by phase contrast and fluorescence microscopy. All ten of the DG5.3-1000Mx-ST26 cell lines were positively stained by the fluorescent lectin, indicating that various cell membrane glycoproteins served as acceptors for the expressed α 2,6-sialyltransferase. In contrast, the FITC-lectin did not stain the parental DG5.3-1000Mx cells.

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9.2.2. CHARACTERIZATION OF RECOMBINANT
HUMAN SECRETED α 2,6-SIALYLATED α -GAL A

Each of the ten DG5.3-1000Mx-ST26 cell lines and the parental DG5.3-1000Mx cells were grown in 100 mm dishes at the same cell density. The amount of secreted human α -Gal A activity per mg protein of total cultured cells was determined for each cell line. The DG5.3-1000Mx-ST26 lines secreted from 28 to 100% of the α -Gal A secreted by the parental cell line (16,000 U/mg protein, Table XI).

20

To determine the percent of the recombinant human secreted α -Gal A that contained α 2,6-sialic acid residues, the secreted enzyme was chromatographed on the immobilized SNA column, and the bound enzyme was eluted with 0.4 M lactose. As shown in Table XI, the individual clones had 55 to 100% of the applied enzymatic activity that was specifically bound and eluted from the lectin column. Notably, clone DG5.3-1000Mx-ST.1 produced the highest amount of recombinant α -Gal A, essentially all the enzyme form was sialylated and bound the immobilized SNA column. In contrast, recombinant α -Gal A secreted by the parental

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DG5.3-1000Mx cells had little, if any binding to α 2,6-sialic acid-specific lectin (Table XI).

5

TABLE XI

DG5.3-1000Mx-ST26 CHO Cell Lines
Secreting Human α 2,6-Sialylated α -Galactosidase A

10	Clone/ Subclone	Percent of Secreted α -Gal A Bound to SNA-Lectin	α -Gal A Secretion (%DG5.3-1000Mx*)
	DG5.3-1000MX	5	100
15	DG5.3-1000Mx-ST26 Subclones:		
	1	100	100
	2	99	76
	3	93	72
	4	79	70
	5	74	64
20	6	73	85
	7	72	85
	8	55	84
	9	51	81
	10	28	79

25

* Activity is expressed as units per mg of cellular protein; The DG5.3-1000Mx line secreted ~16,000 U/mg.

30

9.2.3. PLASMA HALF-LIFE AND TISSUE
DISTRIBUTION OF α 2,6-SIALYLATED
HUMAN α -GAL A IN MICE

35

To determine if the in vivo clearance of recombinant human α 2,6-sialylated α -Gal A was different than that of the non- α 2,6-sialylated enzyme, mice were injected with each form and their circulating half-lives were determined. As shown in

Figure 24A, the presence of α 2,6-sialic acid moieties increased the half-life of the enzyme in the circulation from 14 to almost 30 min. Further, treatment of the α 2,6-sialylated and non- α 2,6-sialylated recombinant enzymes with acid phosphatase increased the in vivo plasma half-life of the non- α 2,6-sialylated enzyme ($T_{1/2}$ from 14 to 24 min), whereas that of the phosphatase-treated α 2,6-sialylated enzyme remained the same at about 28 min (Fig. 24B).

As depicted in Figure 25, the presence or absence of α 2,6-sialic acid moieties and/or phosphate residues, on the secreted recombinant human α -Gal A forms had a marked effect on their respective tissue distributions following intravenous administration to mice. A greater percentage of the total α 2,6-sialylated α -Gal A injected was recovered in the lungs, kidney and heart, as compared to the non- α 2,6-sialylated enzyme; in contrast, less of the α 2,6-sialylated enzyme was recovered in the spleen. Interestingly, when the α 2,6-sialylated enzyme was treated with acid phosphatase, the modified enzyme was redistributed to the liver and spleen, presumably to their reticuloendothelial cells (Fig. 25). Acid phosphatase treatment of the non- α 2,6-sialylated enzyme resulted in the recovery of significantly more enzyme than its non-phosphatase treated form in the lungs and heart whereas, less was recovered in the kidney (Fig. 25).

10. EXAMPLE: OVEREXPRESSION OF HUMAN LYSOSOMAL PROTEINS RESULTS IN THEIR INTRACELLULAR AGGREGATION, CRYSTALLIZATION IN LYSOSOMES, AND SELECTIVE SECRETION

The example presented here describes a method, which is an inherent feature of the invention and was first noted while overexpressing α -Gal A, whereby

certain proteins which are normally intracellularly targeted may be recombinantly expressed in a secreted form. Using several recombinant lysosomal enzymes, the results demonstrate that overexpression of such proteins in CHO cells presumably leads to aggregation which, in turn, causes the protein to be secreted, rather than being targeted to the intracellular vesicles, in this case, lysosomes, to which they would normally be sent. This surprising secretion feature facilitates easy purification of the recombinant protein produced.

10.1. MATERIALS AND METHODS

10.1.1. CONSTRUCTION OF PLASMIDS FOR LYSOSOMAL ENZYME OVERPRODUCTION

Construction of the α -Gal A expression construct, p91-AGA, has been described supra (Section 6.1.2). The analogous expression constructs for human α -N-acetylgalactosaminidase (designated p91-AGB) and acid sphingomyelinase (designated p91-ASM) and the respective transient expression of each in COS-1 cells have been described (Wang et al., 1990, J. Biol. Chem. 265: 21859-21866; Schuchman et al., 1991, J. Biol. Chem. 266: 8535-8539).

10.1.2. CELL CULTURE, ELECTROTRANSFECTION, AND GENE AMPLIFICATION

CHO cells were grown and maintained as described supra, in Section 6.1.3. Likewise, electroporation was performed as described supra, in Section 6.1.3.

Butyrate stimulation of the α -Gal A expressing CHO cells was performed as previously described (Dorner et al., 1989 J. Biol. Chem. 264:20602-20607).

Briefly, cells were plated in 100 mm dishes and allowed to grow in 10 ml of DMEM supplemented with 10%

dFCS for 2 days. The media was removed and replaced with 10 ml of DMEM supplemented with 10% dFCS containing 5 mM sodium butyrate. Cells were incubated for 16 hr at 37 °C in a CO₂ incubator and then cells and culture media were harvested and the α -Gal A activity was determined.

10.1.3. ULTRASTRUCTURAL AND IMMUNOLABELING STUDIES

DG5.3-1000Mx cells were grown to confluency in 100 mm dishes. Following trypsinization (0.25% trypsin, EDTA), they were washed twice in phosphate buffered saline (PBS) and pelleted at 1,500 g for 5 min at room temperature. Cells were then fixed for 1 hr with 3% glutaraldehyde in PBS, followed by fixation in PBS-buffered 1% OsO₄ for 30 min at room temperature. Samples were then dehydrated with graded steps of ethanol, infiltrated with propylene oxide and embedded in Embed 812 (Electron Microscopy Sciences, Fort Washington, PA). 1 μ m sections were cut from representative areas. Ultrathin sections were prepared and stained with uranyl acetate and lead citrate, and then were viewed with an electron microscope (JEM 100 CX, Jeol. USA, Peabody, MA).

For immunodetection, sections were prepared as above, and after embedding, they were mounted on Formvar-coated nickel grids (Formvar Scientific, Marietta, OH), incubated with goat serum in PBS for 30 min at 37°C to block nonspecific binding, washed six times with PBS and then incubated with affinity-purified rabbit anti- α -Gal A antibodies for 1 hr. The sections were washed extensively as above and then were incubated with 10 nm gold particles conjugated to protein A (Amersham Corp., Arlington, IL) for 1 hr at 37°C. After washing with PBS, sections were fixed with 3% glutaraldehyde in PBS for 15 min at room

temperature, washed again with PBS and then examined under the electron microscope.

5 10.1.4. SDS-PAGE AND AUTORADIOGRAPHY

PAGE gel electrophoresis was carried out under reducing conditions as described by Laemmli (1970 Nature (London) 277:680-685) in 1.5 mm thick slab gels containing 10% acrylamide. The gel was fixed in 10%
10 acetic acid and 20% methanol for 30 min and then soaked in Amplify (Amersham Corp.) for 30 min with agitation. Gels were vacuum dried for 90 min (Hoffer Scientific Instruments, San Francisco, CA) and then autoradiographed with Kodak X Omat ARTM film (Eastman
15 Kodak Co.) for 4-24 hrs.

10.1.5. IN VITRO STUDIES OF α -GAL A AGGREGATION

The possible formation of insoluble α -Gal A aggregates at varying enzyme and hydrogen ion
20 concentrations was investigated. Using a stock solution of purified, secreted α -Gal A (16 mg/ml in 10 mM Tris buffer, pH 7.0), appropriate aliquots were placed in glass borosilicate tubes and the volumes were brought to 200 μ l with distilled water so that
25 with the addition of 100 μ l of the appropriate buffer (0.5 M 2-(N-morpholino)ethanesulfonic acid, at pH 5.0, 5.5, 6.0, 6.5 or 7.0), the final α -Gal A concentrations (0.1-10 mg/ml) would be achieved at specific pH values. After incubation for 10 min at
30 room temperature, the turbidity of each solution was determined by measuring the OD at 650 nm in a spectrophotometer (SpectronicTM 1201, Milton Roy Co., Rochester, NY) using a 1-cm path cuvette. As a control, 1 mg/ml of purified, secreted α -Gal A was
35 mixed with increasing BSA concentrations (0.1-10 mg/ml) and the turbidity of each solution was

determined. Similar experiments were performed with solutions of α -Gal A (10 mg/ml) and BSA (2 mg/ml), at decreasing pH (from pH 7.0 to 5.0). After incubation and centrifugation as above, the supernatants and pellets were subjected to SDS-PAGE.

10.1.6. ENZYME AND PROTEIN ASSAYS

The α -Gal A activities in the cell lysates and media were determined using 5 mM 4-methylumbelliferyl- α -D-galactopyranoside (4MU- α -Gal) (Genzyme Corp., Cambridge, MA) as previously described (Bishop and Desnick, 1981 J. Biol. Chem. 256:1307-1316). Briefly, a stock solution of 5 mM 4MU- α -Gal was prepared in 0.1 M citrate/0.2 M phosphate buffer, pH 4.6, in an ultrasonic bath. The reaction mixture, containing 10 - 50 μ l of cell extract and 150 μ l of the stock substrate solution, was incubated at 37°C for 10 to 30 min. The reaction was terminated with the addition of 2.3 ml of 0.1 M ethylenediamine. The fluorescence was determined using a Ratio-2 System Fluorometer (Optical Technology Devices, Elmsford, NY). 1U of activity is the amount of enzyme that hydrolyzed one nmol of substrate per hour. The activities of α -mannosidase, β -galactosidase, β -hexosaminidase, β -glucuronidase, acid phosphatase and α -N-acetylgalactosaminidase were measured using the appropriate 4-methylumbelliferyl substrate. The activity of acid sphingomyelinase was determined according to Gal et al. (1975, N. Eng. J. Med. 293:632-636). Protein concentrations were quantitated by the fluorescamine method (Bohlen et al., 1973, Arch. Biochem. Biophys. 155:213-220) as modified by Bishop et al. (1978, Biochem. Biophys. Acta 524:109-120).

10.2. RESULTS10.2.1. OVEREXPRESSION RESULTS IN CRYSTALLINE
STRUCTURES CONTAINING HUMAN α -GAL A
IN MEMBRANE-LIMITED VESICLES

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Ultrastructural examination of the stably amplified DG5.3-1000Mx cells revealed numerous 0.25 to 1.5 μ m crystalline bodies which had ordered triangular lattices in membrane-limited vesicles throughout the cytoplasm (FIG. 26A and 26B). The repeat within these crystalline structures was about 20 nm. These structures were particularly abundant in lysosomes (FIG. 28A) and in vesicles which appeared to be dilated TGN (FIG. 28B) (Hand and Oliver, 1984, J. Histochem. Cytochem. 42: 403-442; Griffith and Simons, 1986, Science 234:438-443; McCracken, 1991, in Intracellular Trafficking of Proteins, Steer and Hanover, eds., Cambridge University Press, New York pp. 461-485). Of note, normal Golgi structures were not observed in these cells, whereas Golgi complexes were readily identified in the parental DG44 cells (FIG. 26E, inset). When osmium-glutaraldehyde fixed sections of the DG5.3-1000Mx cells were incubated with affinity-purified rabbit anti-human α -Gal A antibodies and then with protein A-conjugated gold, these crystalline structures were specifically stained by gold particles (FIG. 26C and 26D). That the crystalline structures were immunogold labeled, even though the sections were fixed in osmium-glutaraldehyde, suggested that these structures were primarily, if not solely, composed of the human enzyme.

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To determine whether the crystalline structures were present in clones expressing lower levels of α -Gal A, clones DG5.3-0Mx, -1.3Mx, -250Mx, and -1000Mx were grown to confluency and examined by electron

microscopy. Although the TGN was increasingly dilated with increasing α -Gal A expression, only the DG5.3-1000Mx clone contained crystalline arrays in lysosomes. Similarly, clone DG5.3-1.3Mx was stimulated with 5mM sodium butyrate for 18 hrs to increase transcription of the integrated vector containing the α -Gal A cDNA and then examined ultrastructurally. Compared to the untreated clone, butyrate treatment resulted in the presence of dilated organelles including many membrane-bound structures containing dense material. These results indicated that crystal formation was α -Gal A concentration dependent. Furthermore, to assess whether crystal formation was specific to α -Gal A, clone AGB14.8-1000Mx, which overexpresses α -N-Acetylgalactosaminidase, was examined. No crystalline arrays were observed, but numerous dilated structures were seen similar to those in the DG5.3-1.3Mx clone, suggesting that expression of the recombinant α -N-acetylgalactosaminidase had not reached the critical level necessary for crystal formation.

10.2.2. α -GAL A AND α -N-ACETYL GALACTOSAMINIDASE AGGREGATE AT HIGH CONCENTRATION AND LOW pH

Since it was conceivable that the overexpression of α -Gal A resulted in the formation of soluble and particulate aggregates that did not bind to or were inefficiently bound by the M6PR and/or the sulfotransferase in the TGN, the possible aggregation of α -Gal A was assessed in vitro at varying enzyme and hydrogen ion concentrations. As shown in FIG. 27A, the amount of α -Gal A precipitated, compared to about 30% ($>2 \times 10^6$ U) at pH 5.0. At pH 6.0, the estimated pH of the TGN (Griffith and Simons, 1986, Science 234: 438-443), about 12% of the enzyme formed particulate aggregates that could be pelleted by centrifugation at

15,000 x g. FIG. 27B shows that the turbidity, as a measure of aggregation (Halper and Stere, 1977, Arch. Biochem. Biophys. 184: 529-534), of solutions
5 containing 0.1 to 10 mg/ml of α -Gal A at either pH 5.0 or 7.0 increased as a function of enzyme concentration. Moreover, the turbidity of a 1 mg/ml α -Gal A solution was essentially unaffected by the presence of increasing albumin concentrations from 0.1
10 to 10 mg/ml at pH 5.0 (FIG. 27B; control). Finally, electrophoresis of the supernatant and pellet fractions from solutions containing α -Gal A (10 mg/ml) and bovine serum albumin (BSA) (2 mg/ml) incubated at varying hydrogen ion concentrations revealed that the
15 increasing precipitation of α -Gal A with decreasing pH was enzyme specific, as the BSA did not precipitate over this pH range (FIG. 27C).

10.3. DISCUSSION

20 An "aggregation-secretion" model is proposed to account for the rerouting of human α -Gal A as a prototype for overproduced targeted proteins. As depicted in FIG. 28, the overproduced enzyme is normally synthesized and processed until it reaches
25 the trans-Golgi network (TGN). In this structure, the overproduced enzyme is accumulated and subjected to a markedly more acidic environment, (app. pH 6.0), which leads to increased protein-protein interactions that generate soluble and particulate α -Gal A aggregates.
30 As a result of such aggregation, the enzyme's M6P moieties become inaccessible or less accessible for binding to the MGPR. The aggregates with inaccessible MGP moieties, by default, are rerouted via the constitutive secretory pathway (Helms et al., 1990, J.
35 Biol. Chem. 265: 20,027-20,032).

The cellular response, therefore, to the overproduction of lysosome-targeted protein is to transport those proteins having available MGP residues to the lysosome and to reroute the majority of the overproduced, and presumably aggregated, proteins through the constitutive secretion pathway. The fact that large amounts of recombinant human α -Gal A are secreted by CHO cells permits the scaled-up production and easy purification of the recombinant enzyme for crystallography and for trials of enzyme replacement therapy in patients with Fabry disease. In addition, it is clear that the amplification series of overexpressing α -Gal A CHO cells provides a unique experimental mammalian system to efficiently characterize the biosynthesis, posttranslational modifications, and mechanisms responsible for the lysosomal targeting and selective secretion of this prototype lysosomal enzyme, thereby providing further insight into the nature of protein transport and sorting in mammalian cells.

Thus, the overexpression of lysosomal and perhaps other targeted proteins in CHO cells provides a convenient method for producing large amounts of the protein for structural analyses and/or therapeutic applications, as well as providing a useful approach to study protein biosynthesis and sorting.

11. DEPOSIT OF MICROORGANISMS

The following E. coli strains carrying the listed plasmids have been deposited with the Agricultural Research Culture Collection (NRRL), Peoria, IL and have been assigned the following accession number:

	<u>Host Cell</u>	<u>Strain</u>	<u>Plasmid</u>	<u>Accession No.</u>
	E. coli	k12	p91.AGA	B 18722
5	E. Coli	k12	pAGA-PA	B 18723

The present invention is not to be limited in scope by the microorganisms deposited since the deposited embodiments are intended as illustration of individual aspects of the invention and any
10 microorganisms, or constructs which are functionally equivalent are within the scope of this invention. Indeed various modifications of the invention in addition to those shown and described herein will
15 become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Desnick, Robert J.
Bishop, David F.
Ioannou, Yiannis A.
- (ii) TITLE OF INVENTION: Cloning and Expression of Biologically Active alpha-Galactosidase A
- (iii) NUMBER OF SEQUENCES: 13
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: PENNIE & EDMONDS
 - (B) STREET: 1155 Avenue of the Americas
 - (C) CITY: New York
 - (D) STATE: New York
 - (E) COUNTRY: U.S.A.
 - (F) ZIP: 10036
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 07/983,451
 - (B) FILING DATE: 30-NOV-1992
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Coruzzi, Laura A.
 - (B) REGISTRATION NUMBER: 07/983,451
 - (C) REFERENCE/DOCKET NUMBER: 6923-030
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 212-790-9090
 - (B) TELEFAX: 212-869-8864/9741
 - (C) TELEX: 66141 PENNIE

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1393 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 61..1350

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```

AGGTTAATCT TAAAGCCCA GGTTACCCGC GGAAATTTAT GCTGTCCGGT CACCGTGACA      60
ATG CAG CTG AGG AAC CCA GAA CTA CAT CTG GGC TGC GCG CTT GCG CTT      108
Met Gln Leu Arg Asn Pro Glu Leu His Leu Gly Cys Ala Leu Ala Leu
  1             5             10             15
    
```

CGC TTC CTG GCC CTC GTT TCC TGG GAC ATC CCT GGG GCT AGA GCA CTG Arg Phe Leu Ala Leu Val Ser Trp Asp Ile Pro Gly Ala Arg Ala Leu 20 25 30	156
GAC AAT GGA TTG GCA AGG ACG CCT ACC ATG GGC TGG CTG CAC TGG GAG Asp Asn Gly Leu Ala Arg Thr Pro Thr Met Gly Trp Leu His Trp Glu 35 40 45	204
CGC TTC ATG TGC AAC CTT GAC TGC CAG GAA GAG CCA GAT TCC TGC ATC Arg Phe Met Cys Asn Leu Asp Cys Gln Glu Glu Pro Asp Ser Cys Ile 50 55 60	252
AGT GAG AAG CTC TTC ATG GAG ATG GCA GAG CTC ATG GTC TCA GAA GGC Ser Glu Lys Leu Phe Met Glu Met Ala Glu Leu Met Val Ser Glu Gly 65 70 75 80	300
TGG AAG GAT GCA GGT TAT GAG TAC CTC TGC ATT GAT GAC TGT TGG ATG Trp Lys Asp Ala Gly Tyr Glu Tyr Leu Cys Ile Asp Asp Cys Trp Met 85 90 95	348
GCT CCC CAA AGA GAT TCA GAA GGC AGA CTT CAG GCA GAC CCT CAG CGC Ala Pro Gln Arg Asp Ser Glu Gly Arg Leu Gln Ala Asp Pro Gln Arg 100 105 110	396
TTT CCT CAT GGG ATT CGC CAG CTA GCT AAT TAT GTT CAC AGC AAA GGA Phe Pro His Gly Ile Arg Gln Leu Ala Asn Tyr Val His Ser Lys Gly 115 120 125	444
CTG AAG CTA GGG ATT TAT GCA GAT GTT GGA AAT AAA ACC TGC GCA GGC Leu Lys Leu Gly Ile Tyr Ala Asp Val Gly Asn Lys Thr Cys Ala Gly 130 135 140	492
TTC CCT GGG AGT TTT GGA TAC TAC GAC ATT GAT GCC CAG ACC TTT GCT Phe Pro Gly Ser Phe Gly Tyr Tyr Asp Ile Asp Ala Gln Thr Phe Ala 145 150 155 160	540
GAC TGG GGA GTA GAT CTG CTA AAA TTT GAT GGT TGT TAC TGT GAC AGT Asp Trp Gly Val Asp Leu Leu Lys Phe Asp Gly Cys Tyr Cys Asp Ser 165 170 175	588
TTG GAA AAT TTG GCA GAT GGT TAT AAG CAC ATG TCC TTG GCC CTG AAT Leu Glu Asn Leu Ala Asp Gly Tyr Lys His Met Ser Leu Ala Leu Asn 180 185 190	636
AGG ACT GGC AGA AGC ATT GTG TAC TCC TGT GAG TGG CCT CTT TAT ATG Arg Thr Gly Arg Ser Ile Val Tyr Ser Cys Glu Trp Pro Leu Tyr Met 195 200 205	684
TGG CCC TTT CAA AAG CCC AAT TAT ACA GAA ATC CGA CAG TAC TGC AAT Trp Pro Phe Gln Lys Pro Asn Tyr Thr Glu Ile Arg Gln Tyr Cys Asn 210 215 220	732
CAC TGG CGA AAT TTT GCT GAC ATT GAT GAT TCC TGG AAA AGT ATA AAG His Trp Arg Asn Phe Ala Asp Ile Asp Asp Ser Trp Lys Ser Ile Lys 225 230 235 240	780
AGT ATC TTG GAC TGG ACA TCT TTT AAC CAG GAG AGA ATT GTT GAT GTT Ser Ile Leu Asp Trp Thr Ser Phe Asn Gln Glu Arg Ile Val Asp Val 245 250 255	828
GCT GGA CCA GGG GGT TGG AAT GAC CCA GAT ATG TTA GTG ATT GGC AAC Ala Gly Pro Gly Gly Trp Asn Asp Pro Asp Met Leu Val Ile Gly Asn 260 265 270	876
TTT GGC CTC AGC TGG AAT CAG CAA GTA ACT CAG ATG GCC CTC TGG GCT Phe Gly Leu Ser Trp Asn Gln Gln Val Thr Gln Met Ala Leu Trp Ala 275 280 285	924

ATC Ile	ATG Met	GCT Ala	GCT Ala	CCT Pro	TTA Leu	TTC Phe	ATG Met	TCT Ser	AAT Asn	GAC Asp	CTC Leu	CGA Arg	CAC His	ATC Ile	AGC Ser	972
290						295					300					
CCT Pro	CAA Gln	GCC Ala	AAA Lys	GCT Ala	CTC Leu	CTT Leu	CAG Gln	GAT Asp	AAG Lys	GAC Asp	GTA Val	ATT Ile	GCC Ala	ATC Ile	AAT Asn	1020
305					310					315					320	
CAG Gln	GAC Asp	CCC Pro	TTG Leu	GGC Gly	AAG Lys	CAA Gln	GGG Gly	TAC Tyr	CAG Gln	CTT Leu	AGA Arg	CAG Gln	GGA Gly	GAC Asp	AAC Asn	1068
				325				330						335		
TTT Phe	GAA Glu	GTG Val	TGG Trp	GAA Glu	CGA Arg	CCT Pro	CTC Leu	TCA Ser	GGC Gly	TTA Leu	GCC Ala	TGG Trp	GCT Ala	GTA Val	GCT Ala	1116
			340					345					350			
ATG Met	ATA Ile	AAC Asn	CGG Arg	CAG Gln	GAG Glu	ATT Ile	GGT Gly	GGA Gly	CCT Pro	CGC Arg	TCT Ser	TAT Tyr	ACC Thr	ATC Ile	GCA Ala	1164
		355					360					365				
GTT Val	GCT Ala	TCC Ser	CTG Leu	GGT Gly	AAA Lys	GGA Gly	GTG Val	GCC Ala	TGT Cys	AAT Asn	CCT Pro	GCC Ala	TGC Cys	TTC Phe	ATC Ile	1212
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ACA Thr	CAG Gln	CTC Leu	CTC Leu	CCT Pro	GTG Val	AAA Lys	AGG Arg	AAG Lys	CTA Leu	GGG Gly	TTC Phe	TAT Tyr	GAA Glu	TGG Trp	ACT Thr	1260
385					390					395					400	
TCA Ser	AGG Arg	TTA Leu	AGA Arg	AGT Ser	CAC His	ATA Ile	AAT Asn	CCC Pro	ACA Thr	GGC Gly	ACT Thr	GTT Val	TTG Leu	CTT Leu	CAG Gln	1308
				405				410						415		
CTA Leu	GAA Glu	AAT Asn	ACA Thr	ATG Met	CAG Gln	ATG Met	TCA Ser	TTA Leu	AAA Lys	GAC Asp	TTA Leu	CTT Leu	TAAAAAAAAA			1357
			420				425						430			
AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAA															1393	

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 429 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	Gln	Leu	Arg	Asn	Pro	Glu	Leu	His	Leu	Gly	Cys	Ala	Leu	Ala	Leu
1				5					10					15	
Arg	Phe	Leu	Ala	Leu	Val	Ser	Trp	Asp	Ile	Pro	Gly	Ala	Arg	Ala	Leu
		20						25					30		
Asp	Asn	Gly	Leu	Ala	Arg	Thr	Pro	Thr	Met	Gly	Trp	Leu	His	Trp	Glu
		35					40					45			
Arg	Phe	Met	Cys	Asn	Leu	Asp	Cys	Gln	Glu	Glu	Pro	Asp	Ser	Cys	Ile
		50				55					60				
Ser	Glu	Lys	Leu	Phe	Met	Glu	Met	Ala	Glu	Leu	Met	Val	Ser	Glu	Gly
		65			70					75					80
Trp	Lys	Asp	Ala	Gly	Tyr	Glu	Tyr	Leu	Cys	Ile	Asp	Asp	Cys	Trp	Met
				85					90					95	

-103-

Ala Pro Gln Arg Asp Ser Glu Gly Arg Leu Gln Ala Asp Pro Gln Arg
100 105 110

Phe Pro His Gly Ile Arg Gln Leu Ala Asn Tyr Val His Ser Lys Gly
115 120 125

Leu Lys Leu Gly Ile Tyr Ala Asp Val Gly Asn Lys Thr Cys Ala Gly
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Phe Pro Gly Ser Phe Gly Tyr Tyr Asp Ile Asp Ala Gln Thr Phe Ala
145 150 155 160

Asp Trp Gly Val Asp Leu Leu Lys Phe Asp Gly Cys Tyr Cys Asp Ser
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Leu Glu Asn Leu Ala Asp Gly Tyr Lys His Met Ser Leu Ala Leu Asn
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Arg Thr Gly Arg Ser Ile Val Tyr Ser Cys Glu Trp Pro Leu Tyr Met
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Trp Pro Phe Gln Lys Pro Asn Tyr Thr Glu Ile Arg Gln Tyr Cys Asn
210 215 220

His Trp Arg Asn Phe Ala Asp Ile Asp Asp Ser Trp Lys Ser Ile Lys
225 230 235 240

Ser Ile Leu Asp Trp Thr Ser Phe Asn Gln Glu Arg Ile Val Asp Val
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Ala Gly Pro Gly Gly Trp Asn Asp Pro Asp Met Leu Val Ile Gly Asn
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Ile Met Ala Ala Pro Leu Phe Met Ser Asn Asp Leu Arg His Ile Ser
290 295 300

Pro Gln Ala Lys Ala Leu Leu Gln Asp Lys Asp Val Ile Ala Ile Asn
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Gln Asp Pro Leu Gly Lys Gln Gly Tyr Gln Leu Arg Gln Gly Asp Asn
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Phe Glu Val Trp Glu Arg Pro Leu Ser Gly Leu Ala Trp Ala Val Ala
340 345 350

Met Ile Asn Arg Gln Glu Ile Gly Gly Pro Arg Ser Tyr Thr Ile Ala
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Val Ala Ser Leu Gly Lys Gly Val Ala Cys Asn Pro Ala Cys Phe Ile
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Thr Gln Leu Leu Pro Val Lys Arg Lys Leu Gly Phe Tyr Glu Trp Thr
385 390 395 400

Ser Arg Leu Arg Ser His Ile Asn Pro Thr Gly Thr Val Leu Leu Gln
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Leu Glu Asn Thr Met Gln Met Ser Leu Lys Asp Leu Leu
420 425

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

-104-

- (A) LENGTH: 411 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met	Leu	Leu	Lys	Thr	Val	Leu	Leu	Leu	Gly	His	Val	Ala	Gln	Val	Leu	1	5	10	15
Met	Leu	Asp	Asn	Gly	Leu	Leu	Gln	Thr	Pro	Pro	Met	Gly	Trp	Leu	Ala	20	25	30	
Trp	Glu	Arg	Phe	Arg	Cys	Asn	Ile	Asn	Cys	Asp	Glu	Asp	Pro	Lys	Asn	35	40	45	
Cys	Ile	Ser	Glu	Gln	Leu	Phe	Met	Glu	Met	Ala	Asp	Arg	Met	Ala	Gln	50	55	60	
Asp	Gly	Trp	Arg	Asp	Met	Gly	Tyr	Thr	Tyr	Leu	Asn	Ile	Asp	Asp	Cys	65	70	75	80
Trp	Ile	Gly	Gly	Arg	Asp	Ala	Ser	Gly	Arg	Leu	Met	Pro	Asp	Pro	Lys	85	90	95	
Arg	Phe	Pro	His	Gly	Ile	Pro	Phe	Leu	Ala	Asp	Tyr	Val	His	Ser	Leu	100	105	110	
Gly	Leu	Lys	Leu	Gly	Ile	Tyr	Ala	Asp	Met	Gly	Asn	Phe	Thr	Cys	Met	115	120	125	
Gly	Tyr	Pro	Gly	Thr	Thr	Leu	Asp	Lys	Val	Val	Gln	Asp	Ala	Gln	Thr	130	135	140	
Phe	Ala	Glu	Trp	Lys	Val	Asp	Met	Leu	Lys	Leu	Asp	Gly	Cys	Phe	Ser	145	150	155	160
Thr	Pro	Glu	Glu	Arg	Ala	Gln	Gly	Tyr	Pro	Lys	Met	Ala	Ala	Ala	Leu	165	170	175	
Asn	Ala	Thr	Gly	Arg	Pro	Ile	Ala	Phe	Ser	Cys	Ser	Trp	Pro	Ala	Tyr	180	185	190	
Glu	Gly	Gly	Leu	Pro	Pro	Arg	Val	Asn	Tyr	Ser	Leu	Leu	Ala	Asp	Ile	195	200	205	
Cys	Asn	Leu	Trp	Arg	Asn	Tyr	Asp	Asp	Ile	Gln	Asp	Ser	Trp	Trp	Ser	210	215	220	
Val	Leu	Ser	Ile	Leu	Asn	Trp	Phe	Val	Glu	His	Gln	Asp	Ile	Leu	Gln	225	230	235	240
Pro	Val	Ala	Gly	Pro	Gly	His	Trp	Asn	Asp	Pro	Asp	Met	Leu	Leu	Ile	245	250	255	
Gly	Asn	Phe	Gly	Leu	Ser	Leu	Glu	Gln	Arg	Ser	Arg	Ala	Gln	Met	Ala	260	265	270	
Leu	Trp	Thr	Val	Leu	Ala	Ala	Pro	Leu	Leu	Met	Ser	Thr	Asp	Leu	Arg	275	280	285	
Thr	Ile	Ser	Ala	Gln	Asn	Met	Asp	Ile	Leu	Gln	Asn	Pro	Leu	Met	Ile	290	295	300	

-105-

Lys Ile Asn Gln Asp Pro Leu Gly Ile Gln Gly Arg Ile His Lys Glu
 305 310 315 320

Lys Ser Leu Ile Glu Val Tyr Met Arg Pro Leu Ser Asn Lys Ala Ser
 325 330 335

Ala Leu Val Phe Phe Ser Cys Arg Thr Asp Met Pro Tyr Arg Tyr His
 340 345 350

Ser Ser Leu Gly Gln Leu Asn Phe Thr Gly Ser Val Ile Tyr Glu Ala
 355 360 365

Gln Asp Val Tyr Ser Gly Asp Ile Ile Ser Gly Leu Arg Asp Glu Thr
 370 375 380

Asn Phe Thr Val Ile Ile Asn Pro Ser Gly Val Val Met Trp Tyr Leu
 385 390 395 400

Tyr Pro Ile Lys Asn Leu Glu Met Ser Gln Gln
 405 410

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 404 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Phe Ala Phe Tyr Phe Leu Thr Ala Cys Ile Ser Leu Lys Gly Val
 1 5 10 15

Phe Gly Ser Tyr Asn Gly Leu Gly Leu Thr Pro Gln Met Gly Trp Asp
 20 25 30

Asn Trp Asn Thr Phe Ala Cys Asp Val Ser Glu Gln Leu Leu Leu Asp
 35 40 45

Thr Ala Asp Arg Ile Ser Asp Leu Gly Leu Lys Asp Met Gly Tyr Lys
 50 55 60

Tyr Ile Ile Leu Asp Asp Cys Trp Ser Ser Gly Arg Asp Ser Asp Gly
 65 70 75 80

Phe Leu Val Ala Asp Glu Gln Lys Phe Pro Asn Gly Met Gly His Val
 85 90 95

Ala Asp His Leu His Asn Asn Ser Phe Leu Phe Gly Met Tyr Ser Ser
 100 105 110

Ala Gly Glu Tyr Thr Cys Ala Gly Tyr Pro Gly Ser Leu Gly Arg Glu
 115 120 125

Glu Glu Asp Ala Gln Phe Phe Ala Asn Asn Arg Val Asp Tyr Leu Lys
 130 135 140

Tyr Asp Asn Cys Tyr Asn Lys Gly Gln Phe Gly Thr Pro Glu Ser Tyr
 145 150 155 160

Arg Lys Met Ser Asp Ala Leu Asn Lys Thr Gly Arg Pro Ile Phe Tyr
 165 170 175

Ser Cys Asn Trp Gly Leu Tyr Gly Ser Gly Ile Ala Asn Ser Trp Arg
 180 185 190

Met Ser Gly Asp Val Thr Ala Glu Phe Thr Arg Pro Asp Ser Cys Pro
 195 200 205

Asp Gly Tyr Tyr Ala Gly Phe Ser Ile Met Asn Ile Leu Asn Lys Ala
 210 215 220

Ala Pro Met Gly Gln Asn Ala Gly Val Gly Gly Trp Asn Asp Leu Asp
 225 230 235 240

Asn Leu Glu Val Gly Val Gly Asn Leu Thr Asp Asp Glu Glu Lys Ala
 245 250 255

His Phe Ser Met Trp Ala Met Val Lys Ser Pro Leu Ile Ile Gly Ala
 260 265 270

Asn Val Asn Asn Leu Lys Ala Ser Ser Tyr Ser Ile Tyr Ser Gln Ala
 275 280 285

Ser Val Ile Ala Ile Asn Gln Asp Ser Asn Gly Ile Pro Ala Arg Val
 290 295 300

Ser Asp Thr Asp Glu Tyr Gly Glu Ile Trp Ser Gly Pro Leu Asp Asn
 305 310 315 320

Gly Asp Gln Val Val Ala Leu Leu Asn Gly Gly Ser Val Ser Arg Pro
 325 330 335

Met Asn Thr Thr Leu Glu Ile Asp Ser Leu Gly Lys Lys Leu Thr Ser
 340 345 350

Thr Asp Asp Leu Trp Ala Asn Arg Val Thr Ala Ser Ile Gly Arg Lys
 355 360 365

Thr Gly Leu Tyr Glu Tyr Lys Asp Gly Leu Lys Asn Arg Leu Gly Gln
 370 375 380

Lys Gly Ser Leu Ile Leu Asn Val Pro Ala His Ile Ala Phe Arg Leu
 385 390 395 400

Arg Pro Ser Ser

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 12 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Glu Gln Thr Ile Ala Asp Thr Leu Gly Pro Gly Gly
 1 5 10

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 10 amino acids
 - (B) TYPE: amino acid

-107-

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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Pro Ser Val Ile Tyr Gly Asn Val Arg Asn
1          5          10

```

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 13 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```

Glu Val Ala Cys Leu Val Asp Ala Asn Gly Ile Gln Pro
1          5          10

```

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 297 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..279

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```

GAA TGG ACT TCA AGG TTA AGA AGT CAC ATA AAT CCC ACA GGA ACT GTT      48
Glu Trp Thr Ser Arg Leu Arg Ser His Ile Asn Pro Thr Gly Thr Val
1          5          10          15
TTG CTT CAG CTA GAA AAT ACA ATG CAG ATG TCA TTA AAA GAC TTA CTT      96
Leu Leu Gln Leu Glu Asn Thr Met Gln Met Ser Leu Lys Asp Leu Leu
          20          25          30
CCG GCT GGT CCG GCG CAA CAC GAT GAA GCT CAA CAA AAT GCT TTT TAT     144
Pro Ala Gly Pro Ala Gln His Asp Glu Ala Gln Gln Asn Ala Phe Tyr
          35          40          45
CAA GTC TTA AAT ATG CCT AAC TTA AAT GCT GAT CAA CGC AAT GGT TTT     192
Gln Val Leu Asn Met Pro Asn Leu Asn Ala Asp Gln Arg Asn Gly Phe
          50          55          60
ATC CAA AGC CTT AAA GAT GAT CCA AGC CAA AGT GCT AAC GTT TTA GGT     240
Ile Gln Ser Leu Lys Asp Asp Pro Ser Gln Ser Ala Asn Val Leu Gly
65          70          75          80

```

-108-

GAA GCT CAA AAA CTT AAT GAC TCT CAA GCT CCA AAA TAAGGATCCC 286
 Glu Ala Gln Lys Leu Asn Asp Ser Gln Ala Pro Lys
 85 90

GGAATTCGGC C 297

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 92 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Glu Trp Thr Ser Arg Leu Arg Ser His Ile Asn Pro Thr Gly Thr Val
 1 5 10 15
 Leu Leu Gln Leu Glu Asn Thr Met Gln Met Ser Leu Lys Asp Leu Leu
 20 25 30
 Pro Ala Gly Pro Ala Gln His Asp Glu Ala Gln Gln Asn Ala Phe Tyr
 35 40 45
 Gln Val Leu Asn Met Pro Asn Leu Asn Ala Asp Gln Arg Asn Gly Phe
 50 55 60
 Ile Gln Ser Leu Lys Asp Asp Pro Ser Gln Ser Ala Asn Val Leu Gly
 65 70 75 80
 Glu Ala Gln Lys Leu Asn Asp Ser Gln Ala Pro Lys
 85 90

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CCGAATTCAT GCTGTCCGGT CACCGTG 27

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 32 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

-109-

CGCCGGACCA GCCGGAAGTA AGTCTTTTAA TG

32

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CCGGCTGGTC CGGCGCAACA CGATGAAGCT

30

(2) INFORMATION FOR SEQ ID NO:13:

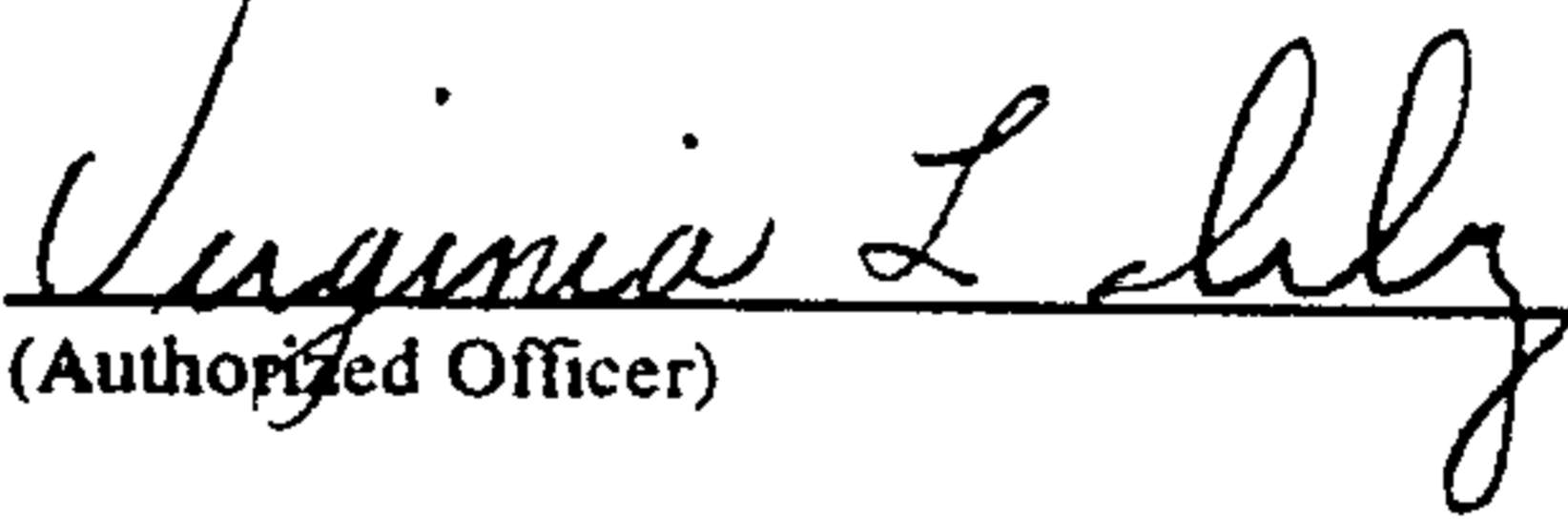
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 36 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGCCGAATTC CGGGATCCTT ATTTGGAGC TTGAGA

36

MICROORGANISMS	
Optional Sheet in connection with the microorganism referred to on page 98, lines 29-33 of the description *	
A. IDENTIFICATION OF DEPOSIT *	
Further deposits are identified on an additional sheet *	
Name of depositary institution *	
Agricultural Research Culture Collection (NRRL) International Depository Authority	
Address of depositary institution (including postal code and country) *	
1815 N. University Street Peoria, IL 61604 US	
Date of deposit * <u>October 5, 1990</u> Accession Number * <u>B 18722</u>	
B. ADDITIONAL INDICATIONS * (leave blank if not applicable). This information is continued on a separate attached sheet	
C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE * (if the indications are not all designated States)	
D. SEPARATE FURNISHING OF INDICATIONS * (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g., "Accession Number of Deposit")	
E. <input checked="" type="checkbox"/> This sheet was received with the International application when filed (to be checked by the receiving Office)	
 _____ (Authorized Officer)	
<input type="checkbox"/> The date of receipt (from the applicant) by the International Bureau *	
was	_____
	(Authorized Officer)

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-111-

International Application No: PCT/ /

Form PCT/RO/134 (cont.)

Agricultural Research Culture Collection (NRRL)
International Depository Authority

1815 N. University Street
Peoria, IL 61604
US

Accession No.

B 18723

Date of Deposit

October 5, 1990

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The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A method for producing a secreted human α -galactosidase A, comprising:
 - (a) amplifying an α -galactosidase A nucleotide sequence in an engineered mammalian cell, wherein said cell expresses α -galactosidase A and dihydrofolate reductase (DHFR), by step-wise growth in increasing methotrexate concentrations so that the α -galactosidase A is sufficiently overexpressed by said cell so as to form crystalline structures,
 - (b) culturing said cell under conditions in which the α -galactosidase A is selectively secreted into the mammalian cell culture medium; and
 - (c) isolating said α -galactosidase A from the cell culture medium.
2. The method according to claim 1, wherein the sufficient overexpression is achieved by a combination of (i) amplification of the α -galactosidase A nucleotide sequence and (ii) butyrate stimulation.
3. The method according to claim 1 or claim 2, wherein said α -galactosidase A contains mannose-6-phosphate
4. The method according to any one of claims 1 to 3, wherein said α -galactosidase A is sialylated.
5. The method according to claim 4, wherein said α -galactosidase A is α 2,6-sialylated α -galactosidase A.

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6. A method for producing a secreted human $\alpha 2,6$ -sialylated α -galactosidase A, comprising:

- (a) culturing an engineered mammalian cell expressing $\alpha 2,6$ -sialyltransferase and α -galactosidase A under conditions in which the α -galactosidase A is overexpressed and selectively secreted into the mammalian cell culture medium; and
- (b) isolating said α -galactosidase A from the cell culture medium.

7. The method according to claim 6, wherein said cell expresses dihydrofolate reductase (DHFR), and wherein said α -galactosidase A in the cell culture medium is obtainable by amplifying an α -galactosidase A nucleotide sequence with 1000 μ M methotrexate.

8. The method according to claim 6 or claim 7, wherein said α -galactosidase A contains mannose-6-phosphate.

9. A secreted human $\alpha 2,6$ -sialylated α -galactosidase A containing mannose-6-phosphate.

10. The α -galactosidase A of claim 9, obtainable by the method of claim 5 or 8.

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AGGTTA

ATCT TAAAAGCCCA GGTTACCCGC GGAAATTTAT GCTGTCCGGT CACCGTGACA -1

1 ATG CAG CTG AGG AAC CCA GAA CTA CAT CTG GGC TGC GCG CTT GCG
 1 Met Gln Leu Arg Asn Pro Glu Leu His Leu Gly Cys Ala Leu Ala

CTT CGC TTC CTG GCC CTC GTT TCC TGG GAC ATC CCT GGG GCT AGA 90
 Leu Arg Phe Leu Ala Leu Val Ser Trp Asp Ile Pro Gly Ala Arg 30

91 GCA CTG GAC AAT GGA TTG GCA AGG ACG CCT ACC ATG GGC TGG CTG
 31 Ala Leu Asp Asn Gly Leu Ala Arg Thr Pro Thr Met Gly Trp Leu
 N-Ter

CAC TGG GAG CGC TTC ATG TGC AAC CTT GAC TGC CAG GAA GAG CCA 180
 His Trp Glu Arg Phe Met Cys Asn Leu Asp Cys Gln Glu Glu Pro 60
 Ser Arg

181 GAT TCC TGC ATC AGT GAG AAG CTC TTC ATG GAG ATG GCA GAG CTC
 61 Asp Ser Cys Ile Ser Glu Lys Leu Phe Met Glu Met Ala Glu Leu
 X X Ser

ATG GTC TCA GAA GGC TGG AAG GAT GCA GGT TAT GAG TAC CTC TGC 270
 Met Val Ser Glu Gly Trp Lys Asp Ala Gly Tyr Glu Tyr Leu Cys 90

271 ATT GAT GAC TGT TGG ATG GCT CCC CAA AGA GAT TCA GAA GGC AGA
 91 Ile Asp Asp Cys Trp Met Ala Pro Gln Arg Asp Ser Glu Gly Arg

CTT CAG GCA GAC CCT CAG CGC TTT CCT CAT GGG ATT CGC CAG CTA 360
 Leu Gln Ala Asp Pro Gln Arg Phe Pro His Gly Ile Arg Gln Leu 120

361 GCT AAT TAT GTT CAC AGC AAA GGA CTG AAG CTA GGG ATT TAT GCA
 121 Ala Asn Tyr Val His Ser Lys Gly Leu Lys Leu Gly Ile Tyr Ala

GAT GTT GGA AAT AAA ACC TGC GCA GGC TTC CCT GGG AGT TTT GGA 450
 Asp Val Gly Asn Lys Thr Cys Ala Gly Phe Pro Gly Ser Phe Gly 150
 CHD -----

451 TAC TAC GAC ATT GAT GCC CAG ACC TTT GCT GAC TGG GGA GTA GAT
 151 Tyr Tyr Asp Ile Asp Ala Gln Thr Phe Ala Asp Trp Gly Val Asp

CTG CTA AAA TTT GAT GGT TGT TAC TGT GAC AGT TTG GAA AAT TTG 540
 Leu Leu Lys Phe Asp Gly Cys Tyr Cys Asp Ser Leu Glu Asn Leu 180

FIG.1A

RECTIFIED SHEET (RULE 91)

2/38

541 GCA GAT GGT TAT AAG CAC ATG TCC TTG GCC CTG AAT AGG ACT GGC
 181 Ala Asp Gly Tyr Lys His Met Ser Leu Ala Leu Asn Arg Thr Gly
 CHD -----

AGA AGC ATT GTG TAC TCC TGT GAG TGG CCT CTT TAT ATG TGG CCC 630
 Arg Ser Ile Val Tyr Ser Cys Glu Trp Pro Leu Tyr Met Trp Pro 210

631 TTT CAA AAG CCC AAT TAT ACA GAA ATC CGA CAG TAC TGC AAT CAC
 211 Phe Gln Lys Pro Asn Tyr Thr Glu Ile Arg Gln Tyr Cys Asn His
 CHD -----

TGG CGA AAT TTT GCT GAC ATT GAT GAT TCC TGG AAA AGT ATA AAG 720
 Trp Arg Asn Phe Ala Asp Ile Asp Asp Ser Trp Lys Ser Ile Lys 240
 Asn X

T-49 _____

721 AGT ATC TTG GAC TGG ACA TCT TTT AAC CAG GAG AGA ATT GTT GAT
 241 Ser Ile Leu Asp Trp Thr Ser Phe Asn Gln Glu Arg Ile Val Asp

GTT GCT GGA CCA GGG GGT TGG AAT GAC CCA GAT ATG TTA GTG ATT 810
 Val Ala Gly Pro Gly Gly Trp Asn Asp Pro Asp Met Leu Val Ile 270

811 GGC AAC TTT GGC CTC AGC TGG AAT CAG CAA GTA ACT CAG ATG GCC
 271 Gly Asn Phe Gly Leu Ser Trp Asn Gln Gln Val Thr Gln Met Ala

CTC TGG GCT ATC ATG GCT GCT CCT TTA TTC ATG TCT AAT GAC CTC 900
 Leu Trp Ala Ile Met Ala Ala Pro Leu Phe Met Ser Asn Asp Leu 300
 CB-1 _____

901 CGA CAC ATC AGC CCT CAA GCC AAA GCT CTC CTT CAG GAT AAG GAC
 301 Arg His Ile Ser Pro Gln Ala Lys Ala Leu Leu Gln Asp Lys Asp
 X X

T-53B _____

GTA ATT GCC ATC AAT CAG GAC CCC TTG GGC AAG CAA GGG TAC CAG 990
 Val Ile Ala Ile Asn Gln Asp Pro Leu Gly Lys Gln Gly Tyr Gln 330
 Arg Glu

FIG.1B

RECTIFIED SHEET (RULE 91)

991 CTT AGA CAG GGA GAC AAC TTT GAA GTG TGG GAA CGA CCT CTC TCA
 331 Leu Arg Gln Gly Asp Asn Phe Glu Val Trp Glu Arg Pro Leu Ser
 Leu Gly Ser Lys X

T-88

GGC TTA GCC TGG GCT GTA GCT ATG ATA AAC CGG CAG GAG ATT GGT 1080
 Gly Leu Ala Trp Ala Val Ala Met Ile Asn Arg Gln Glu Ile Gly 360

1081 GGA CCT CGC TCT TAT ACC ATC GCA GTT GCT TCC CTG GGT AAA GGA
 361 Gly Pro Arg Ser Tyr Thr Ile Ala Val Ala Ser Leu Gly Lys Gly

GTG GCC TGT AAT CCT GCC TGC TTC ATC ACA CAG CTC CTC CCT GTG 1170
 Val Ala Cys Asn Pro Ala Cys Phe Ile Thr Gln Leu Leu Pro Val 390

1171 AAA AGG AAG CTA GGG TTC TAT GAA TGG ACT TCA AGG TTA AGA AGT
 391 Lys Arg Lys Leu Gly Phe Tyr Glu Trp Thr Ser Arg Leu Arg Ser

T-51

T-53A

CAC ATA AAT CCC ACA GGC ACT GTT TTG CTT CAG CTA GAA AAT ACA 1260
 His Ile Asn Pro Thr Gly Thr Val Leu Leu Gln Leu Glu Asn Thr 420
 CHD -----

1261 ATG CAG ATG TCA TTA AAA GAC TTA CTT TAA AAAAAAAAAA AAAAAAAAAA
 421 Met Gln Met Ser Leu Lys Asp Leu Leu Ter 429

AAAAAAAAAA AAAAAAAAAA AAA 1333

FIG.1C

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CONT.
ON
FIG.1E

FIG.1D

```

Go1 B: 1  L D N G L L Q T P P M G W L A W E R F R C N I N C D E D P K N C I S E Q L F M E M A D R M A Q
Go1 A: 1  L D N G L A R T P T M G W L H W E R F M C N L D C Q E E P D S C I S E K L F M E M A E L M V S
Me1 I: 1  L S Y N G L G L T P Q M G W D N W N T F A C D V S E Q L L D T A D R I S D

Go1 B: 117  G I Y A D M G N F T C M G Y P G T T L D K V V Q D A Q T F A E W K V D M L K L D G C F S T P E
Go1 A: 132  G I Y A D V G N K T C A G F P G S F G Y D I D A Q T F A D W G V D L L K F D G C Y C D S L
Me1 I: 108  G M Y S S A G E Y T C A G Y P G S L G R E E E D A Q F F A N N R V D Y L K Y D N C Y N K G Q

Go1 B: 216  D D I Q D S W S V L S I L N W F V E H Q D I L Q P V A G P G H W N D P D M I L L I G N F G L S
Go1 A: 230  A D I D S W K S I K S I L D W T S F N Q E R I V D V A G P G G W N D P D M L V I G N F G L S
Me1 I: 205  D S C P D G Y Y A G F S I M N I L N K A A P M G Q N A G V G G W N D L D N L E V G V G N L T
Me1 A: 108  E Q T I A D T L G P G G I 19

Go1 B: 316  R I H K E K S L I E V Y M R P L S N K A S A L V F F S C R T D M P Y R Y H S S L G Q L N
Go1 A: 329  Y Q L R Q G D N F E V W E R P L S G L A W A V A M I N R Q E I G G P R S Y T I A V A S L G K
Me1 I: 303  I R I V S D T D E Y G E I I W S G P L D N G D Q V V A L L N G G S V S R P P M N T T L E I I D S I L G I K
    
```

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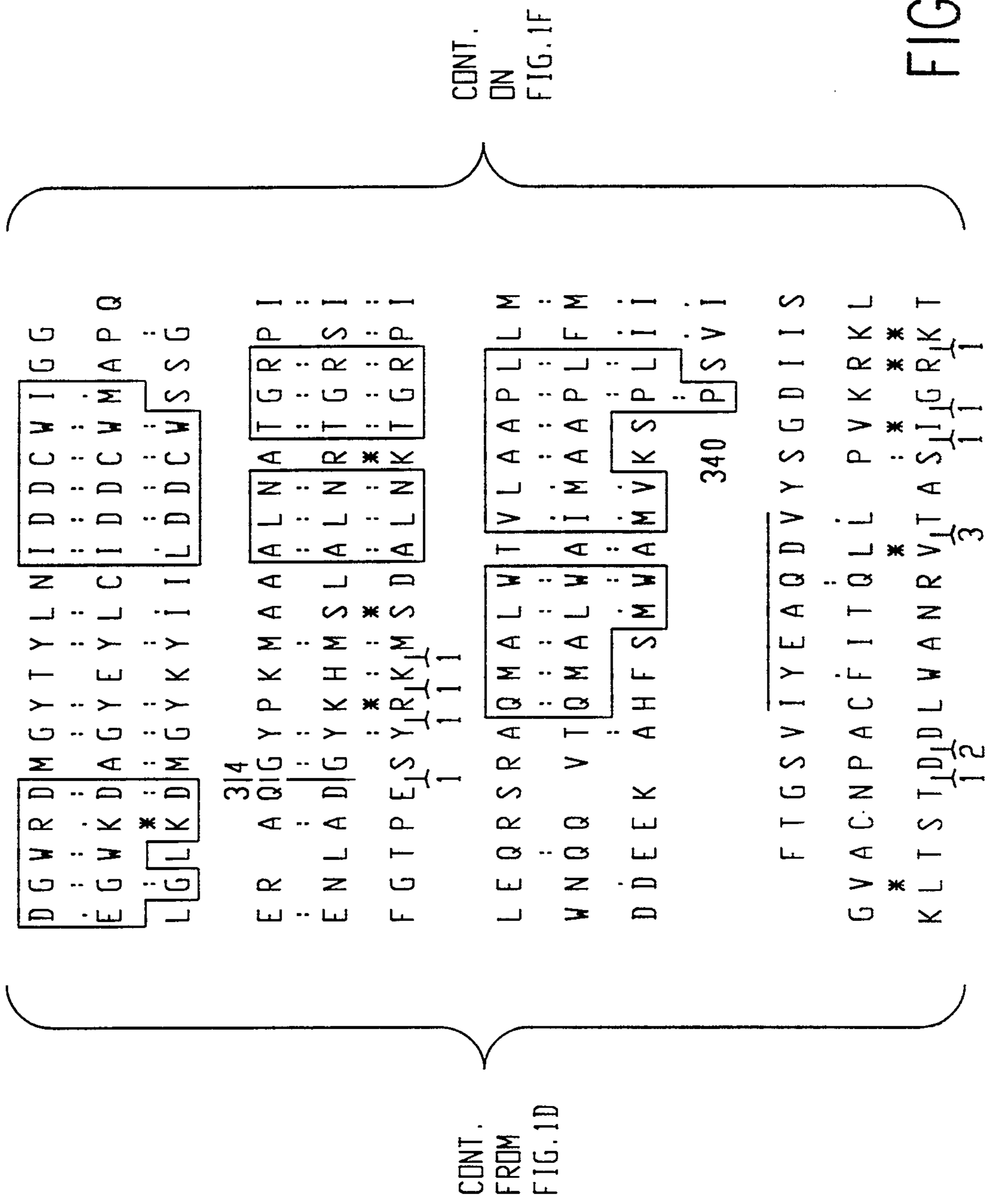


FIG. 1E


```

      MLLKTVLLLGHVAQVLM 17
      MQLRNPELHLCALALRFLALVSWDIPGARA 31
      MFAFYFLTACISLKGVFG 18
      RDASGRLMPPDKRFFPHGIPFFLADYIVHSLGLKL 116
      : : : : : : : : : : : : : : : :
      RDSERLQADPQRFPHGIRQLANRYVHSKGLKL 131
      : * : * : * : * : * : * : * : * :
      RSDGFLVADEQKFPNGMGHVADHLLHNNSLF 107

      AFSCSWPAYEGGLPPRIVNYSLLADICNLWRNY 215
      : : : : : : : : : : : : : : :
      VYSCWPLYMWPFGKPNYTEIRQYCNHWRNF 229
      * : : : : : : : : : : : : : :
      FYSJCNWGLLYGSJGIAN SWRMSGDVTAEFTRP 204
      : : : : : : : : : : : : : :
      STDLRTISAQNM DILQNP LMIKINQDPLGIQG 315
      : : : : : : : : : : : : : :
      SNDLRHISPQAKALLQDKDVAIAINQDPLGKQG 328
      : : : : : : : : : : : : : :
      GANVN NLKASSYSIYSQASVIAINQDSNGIPA 302
      * * : * : * : * : * : * : * :
      YGNVRN 349 363 EVA CLVDANGIQP 375

      GLRDETN FTVIINPSGVVMWYLYPIKNLEMSQQ 411
      : : : : : : : : : : : : : :
      GFEWTSRLRS HINPTGTVL LQLENTMNSLKDLL 429
      : * * : * : * : * : * : * : * :
      IGLYIEYKDG LK N R LG QK G S LJ L N V P A H J I A F I R L R P S S 404
      : : : : : : : : : : : : : :

```

FIG.1F

CONT.
FROM
FIG.1E

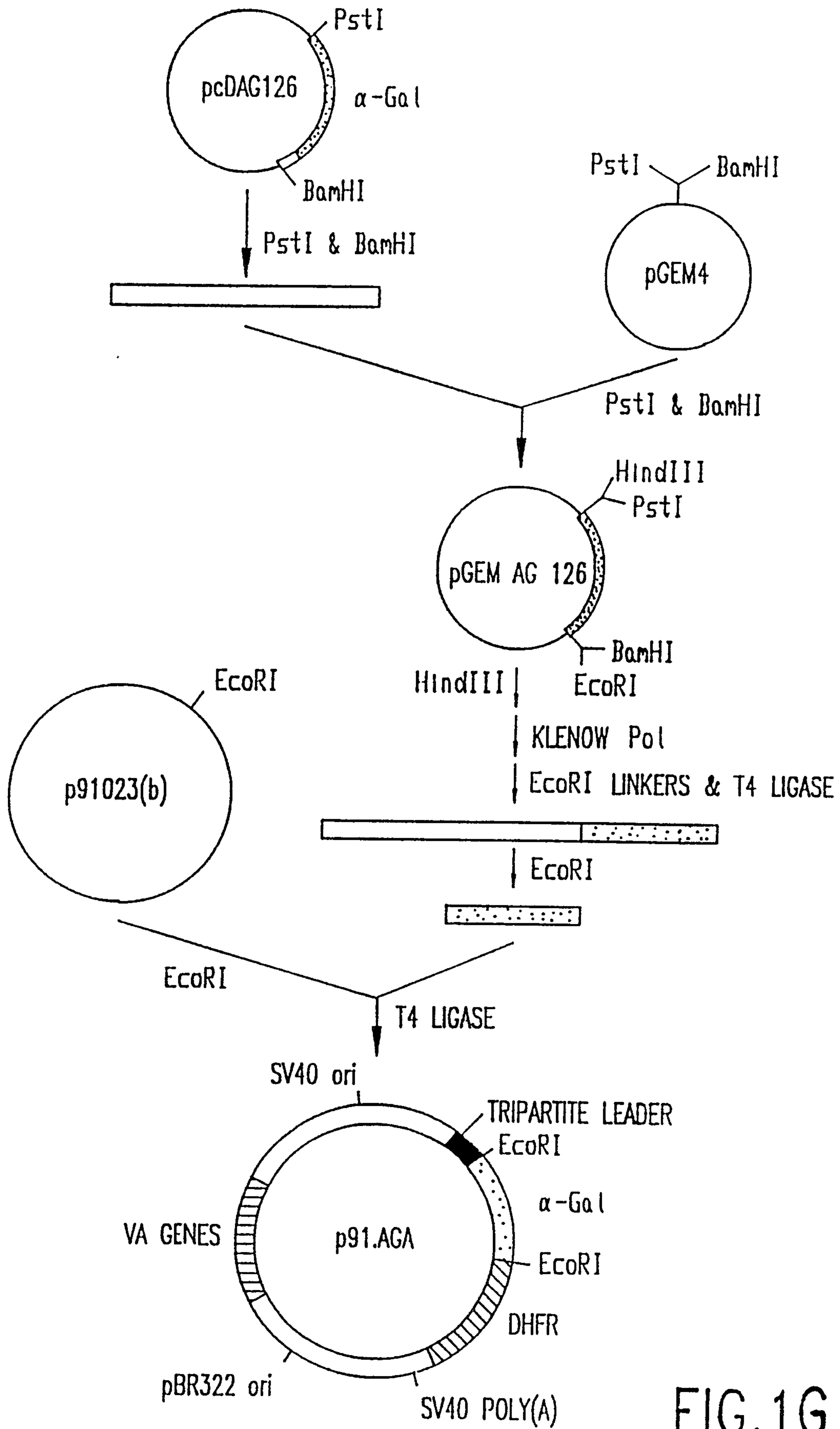


FIG.1G

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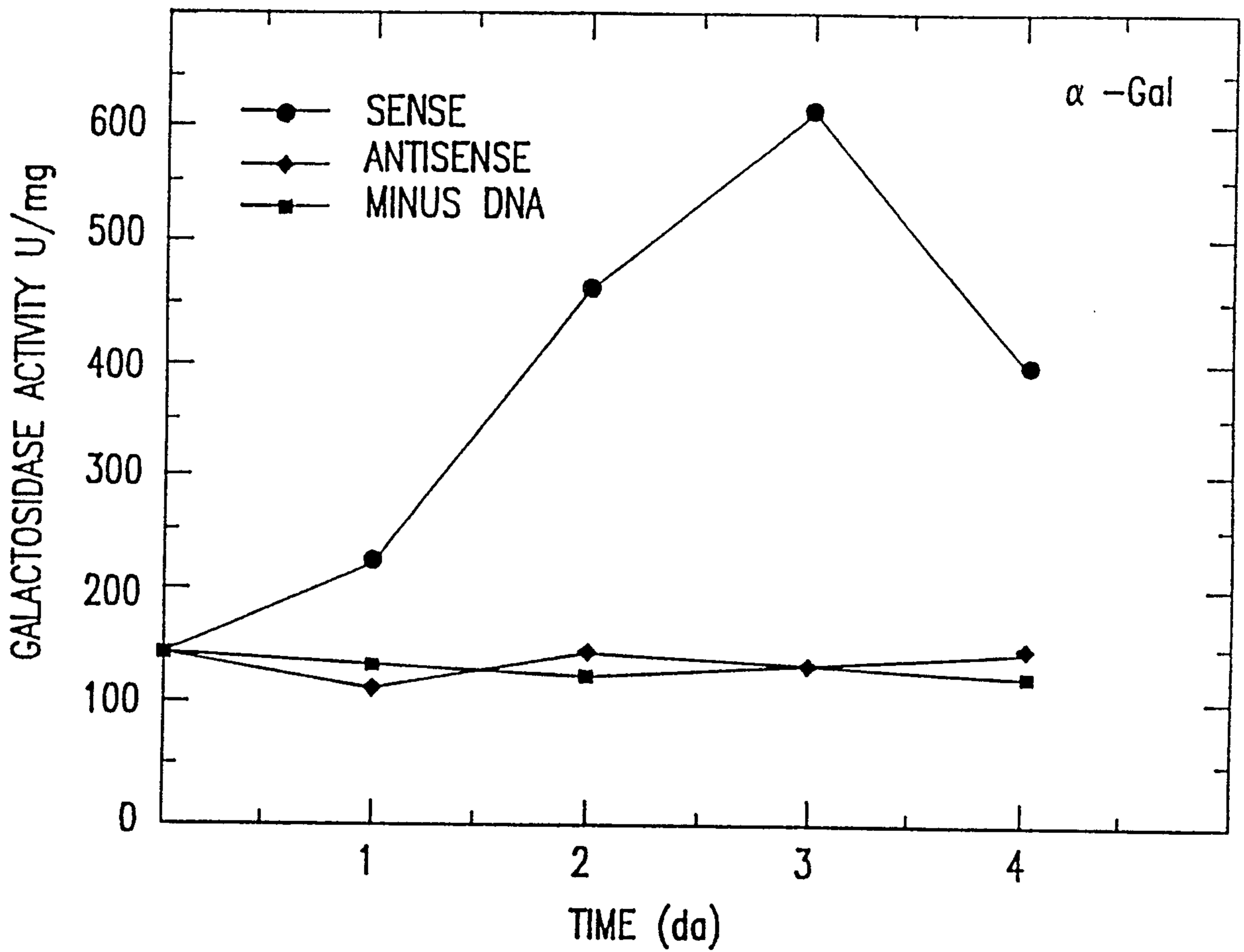


FIG.2A

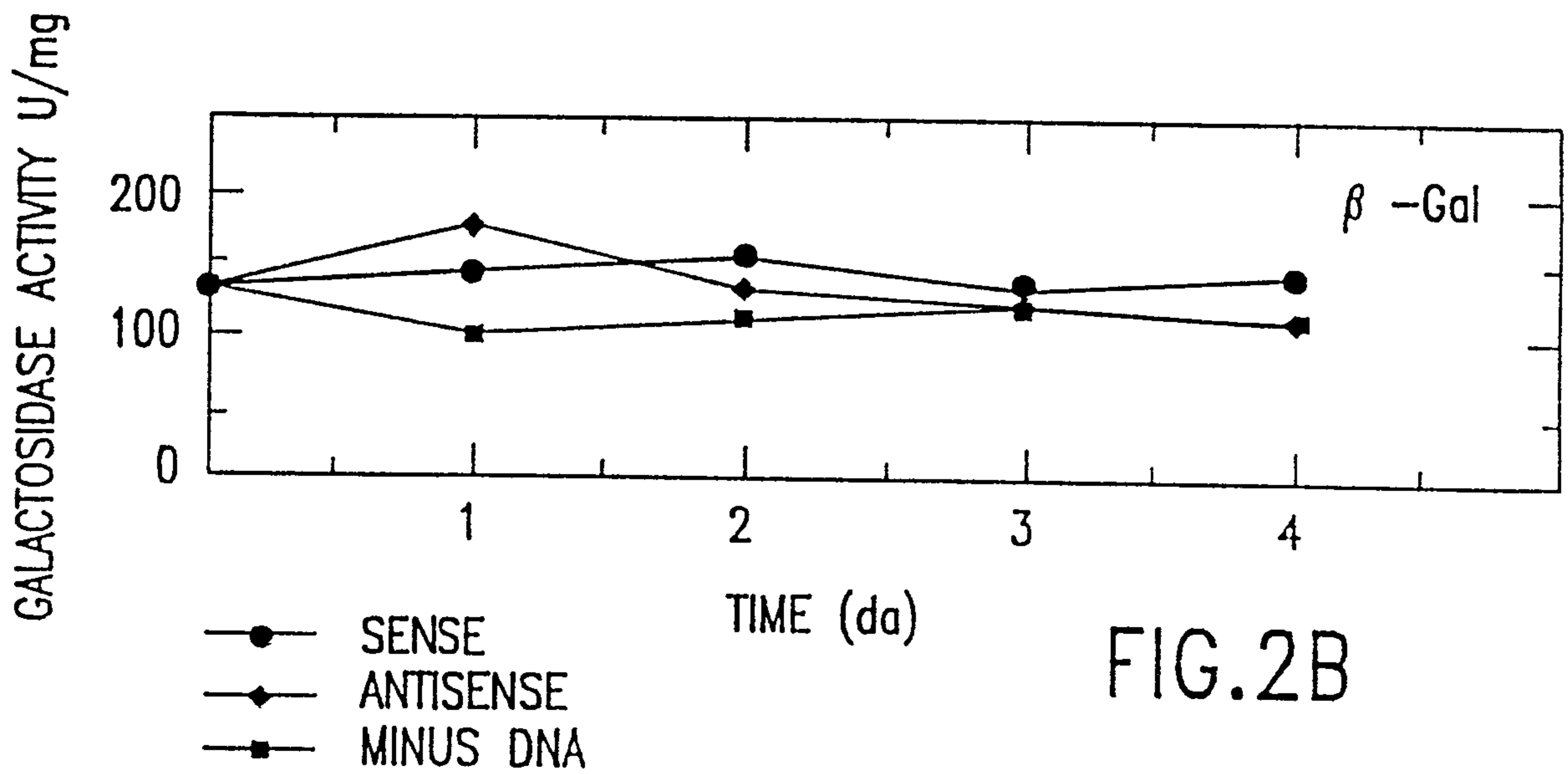


FIG.2B

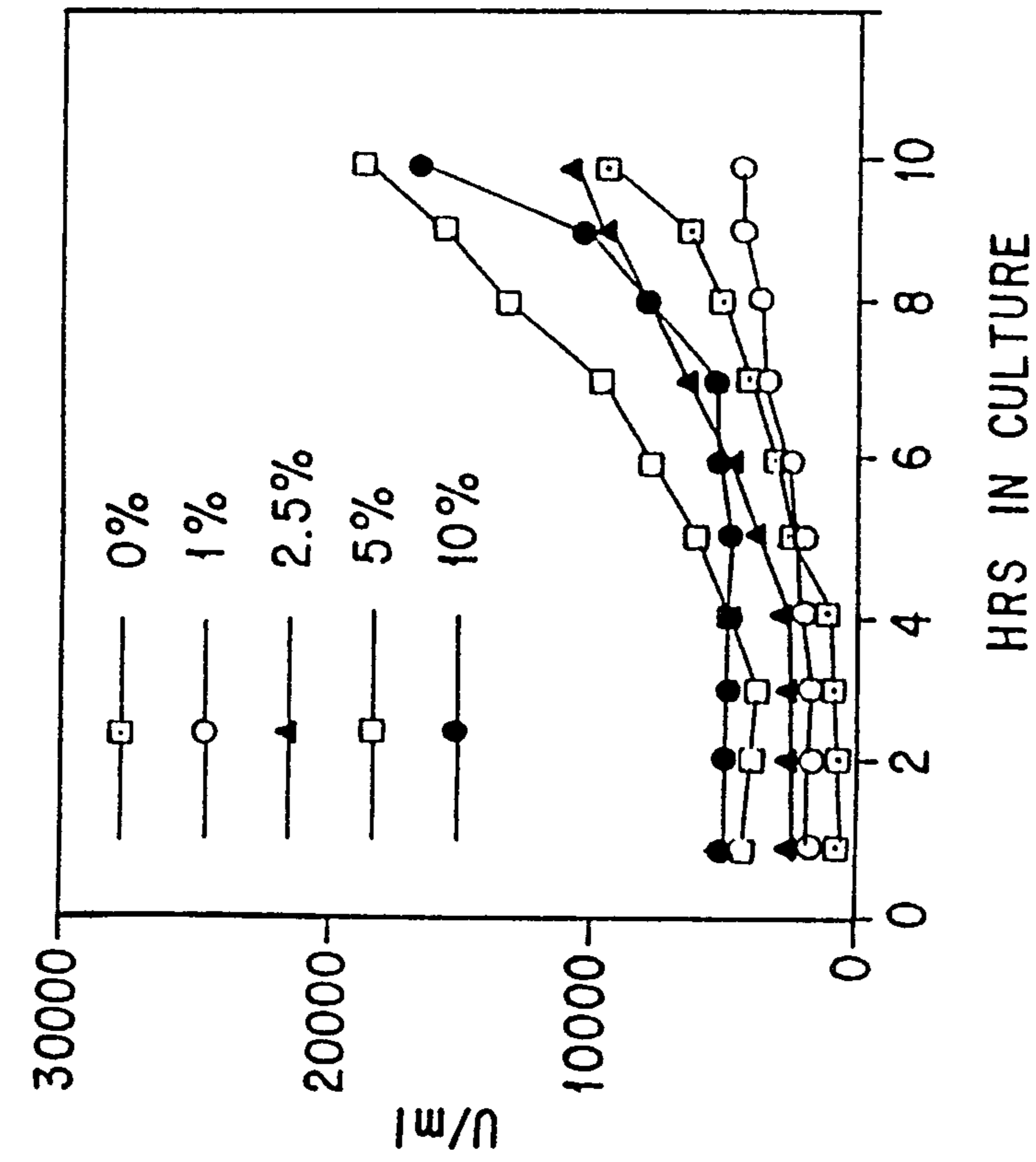


FIG. 3B

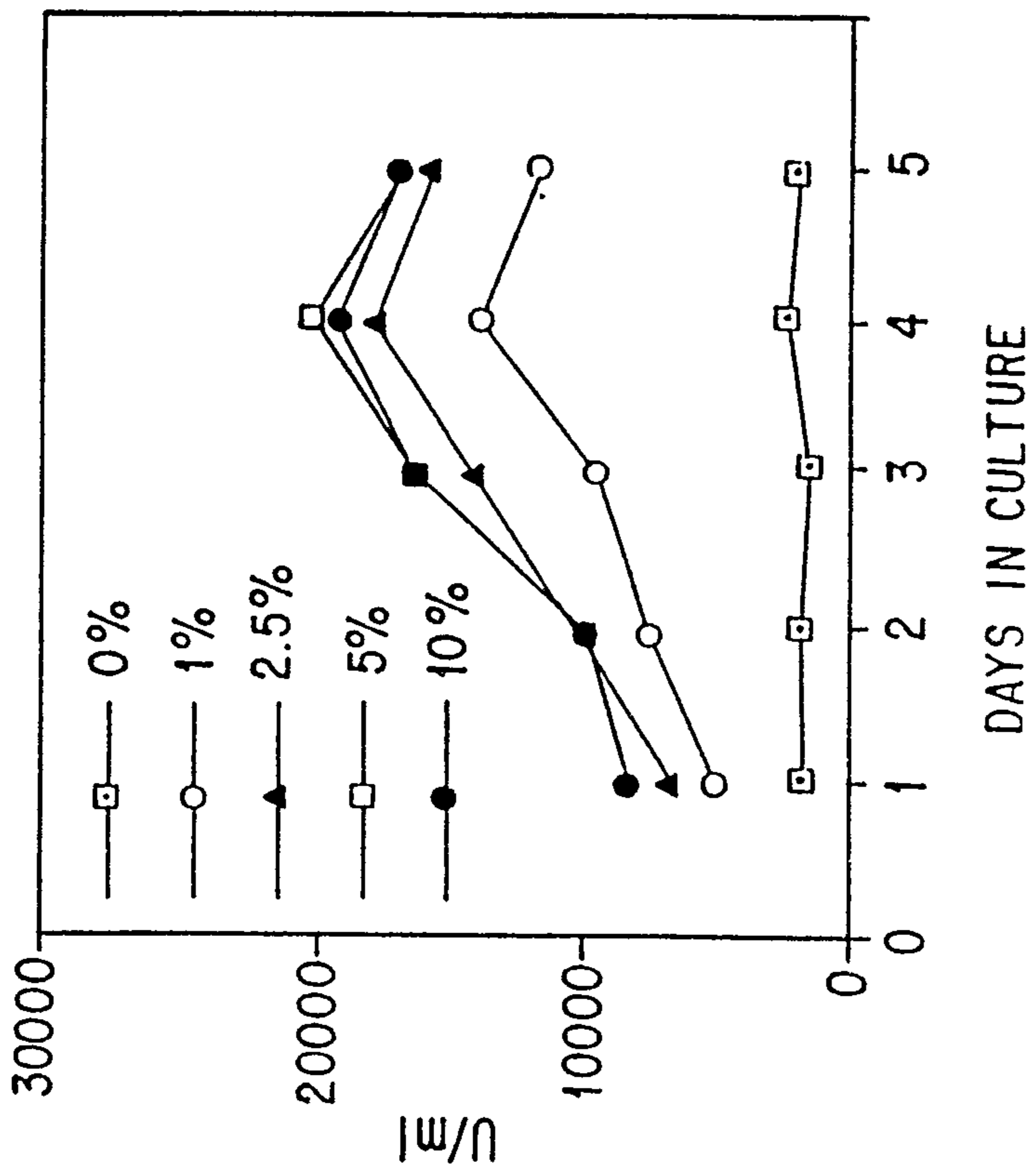


FIG. 3A

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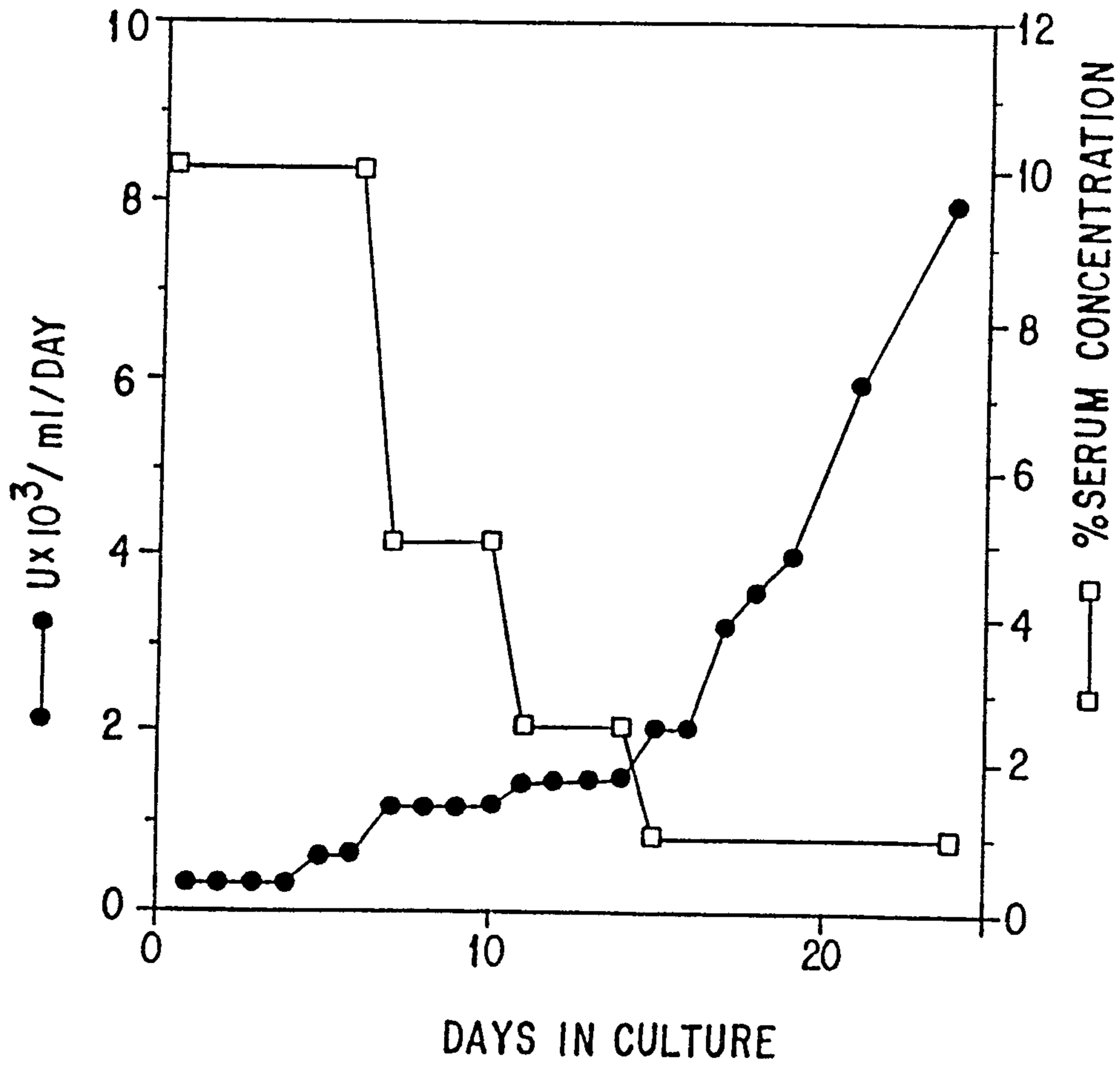


FIG. 4

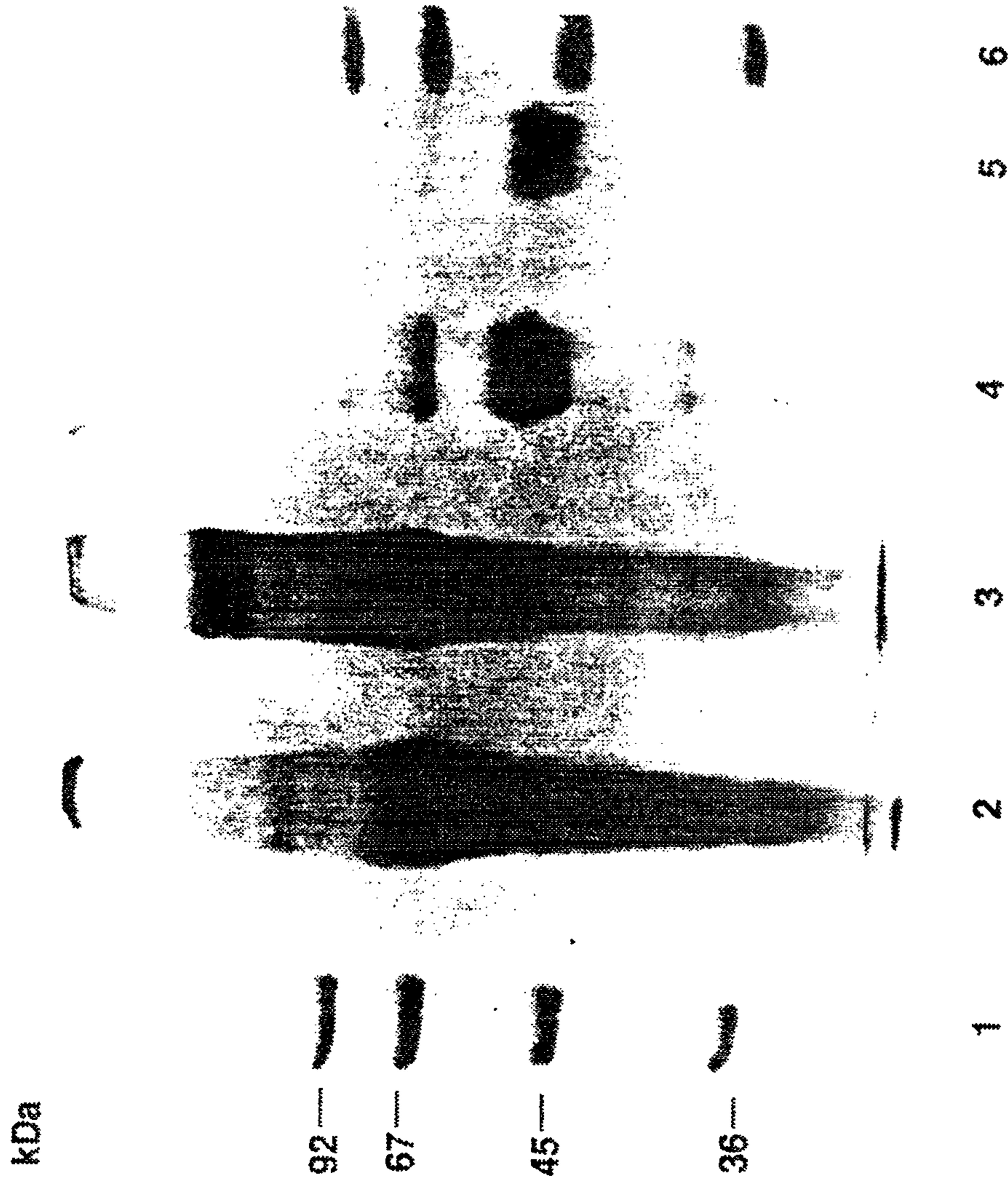


FIG. 5

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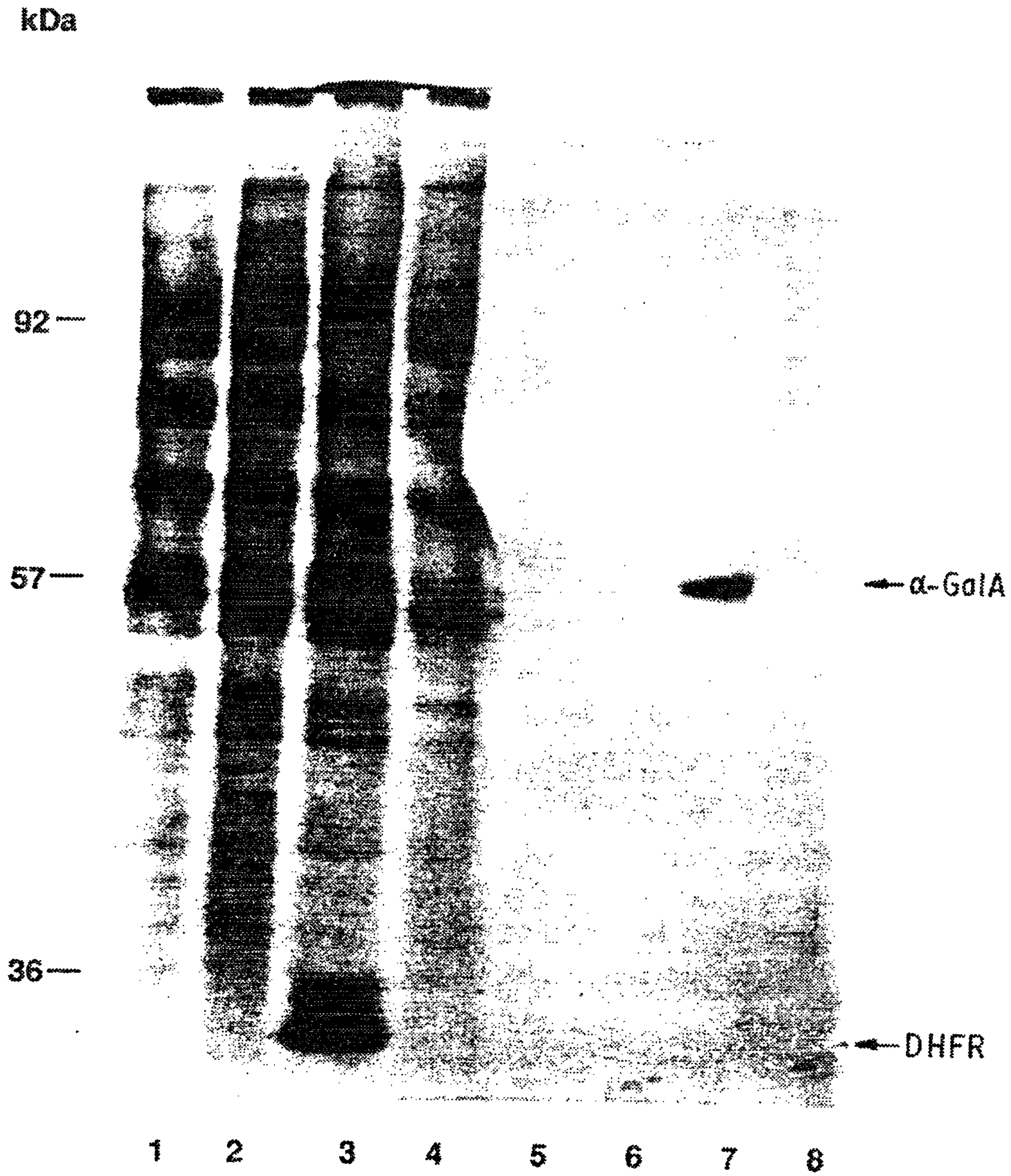


FIG. 6

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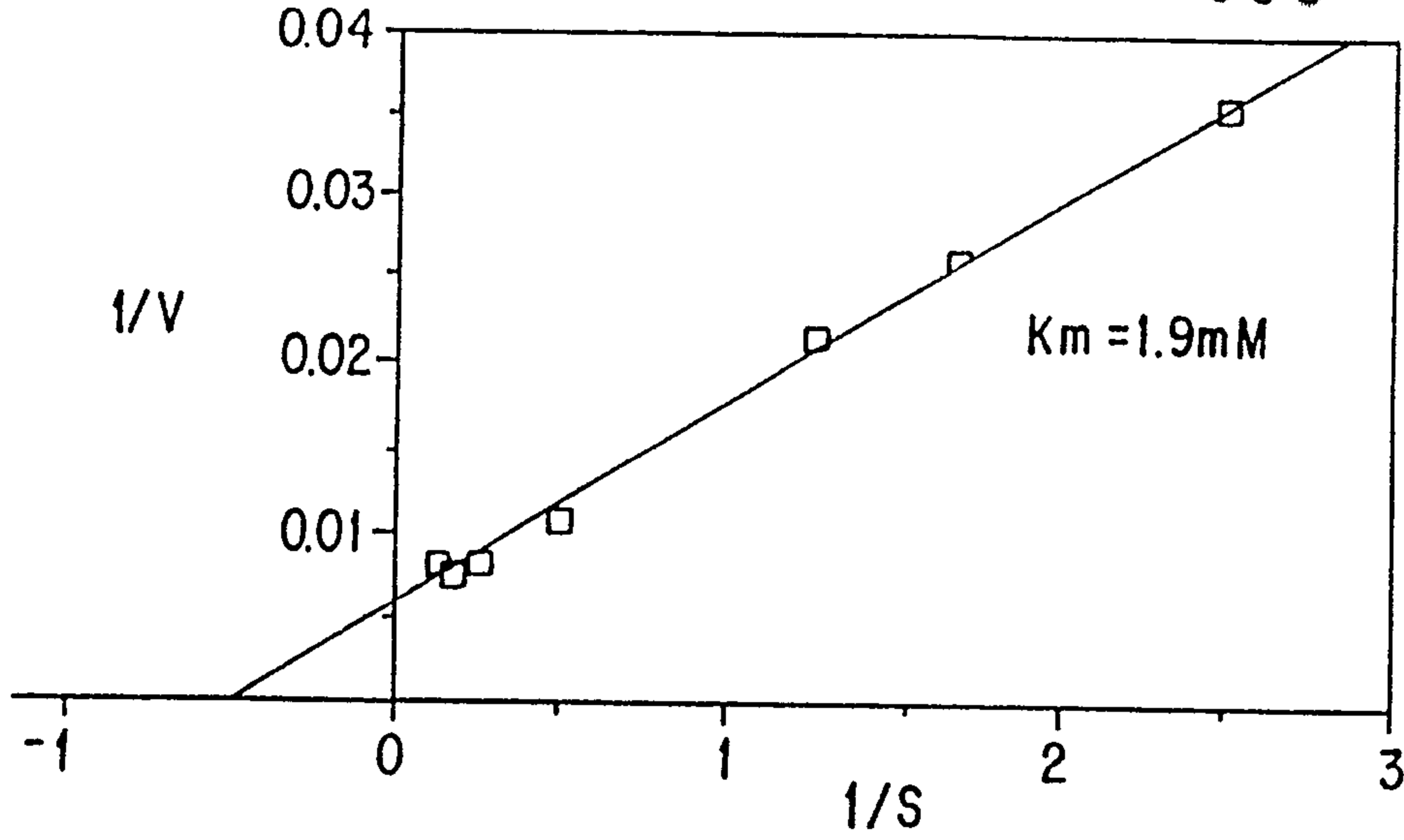


FIG.7A

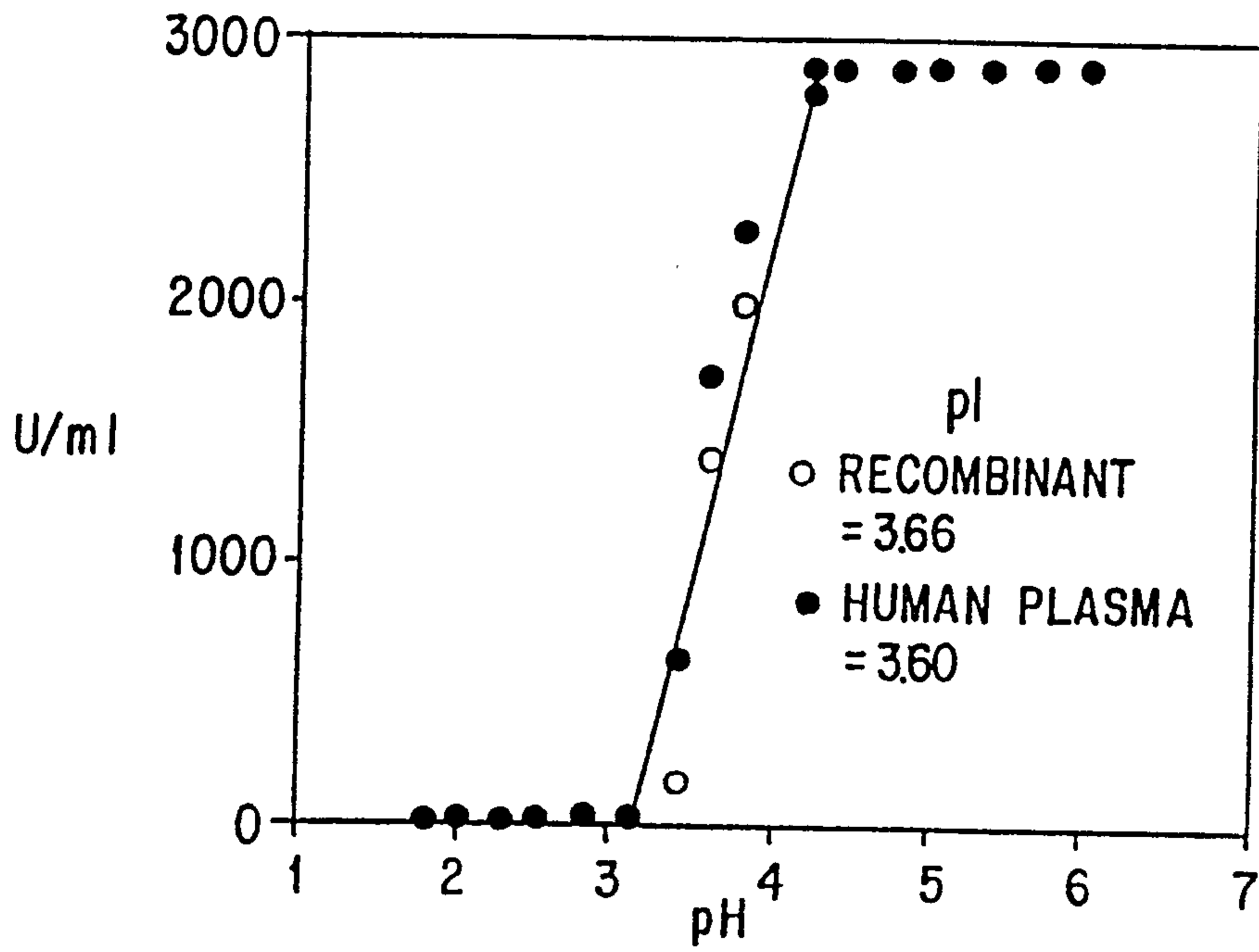


FIG.7B

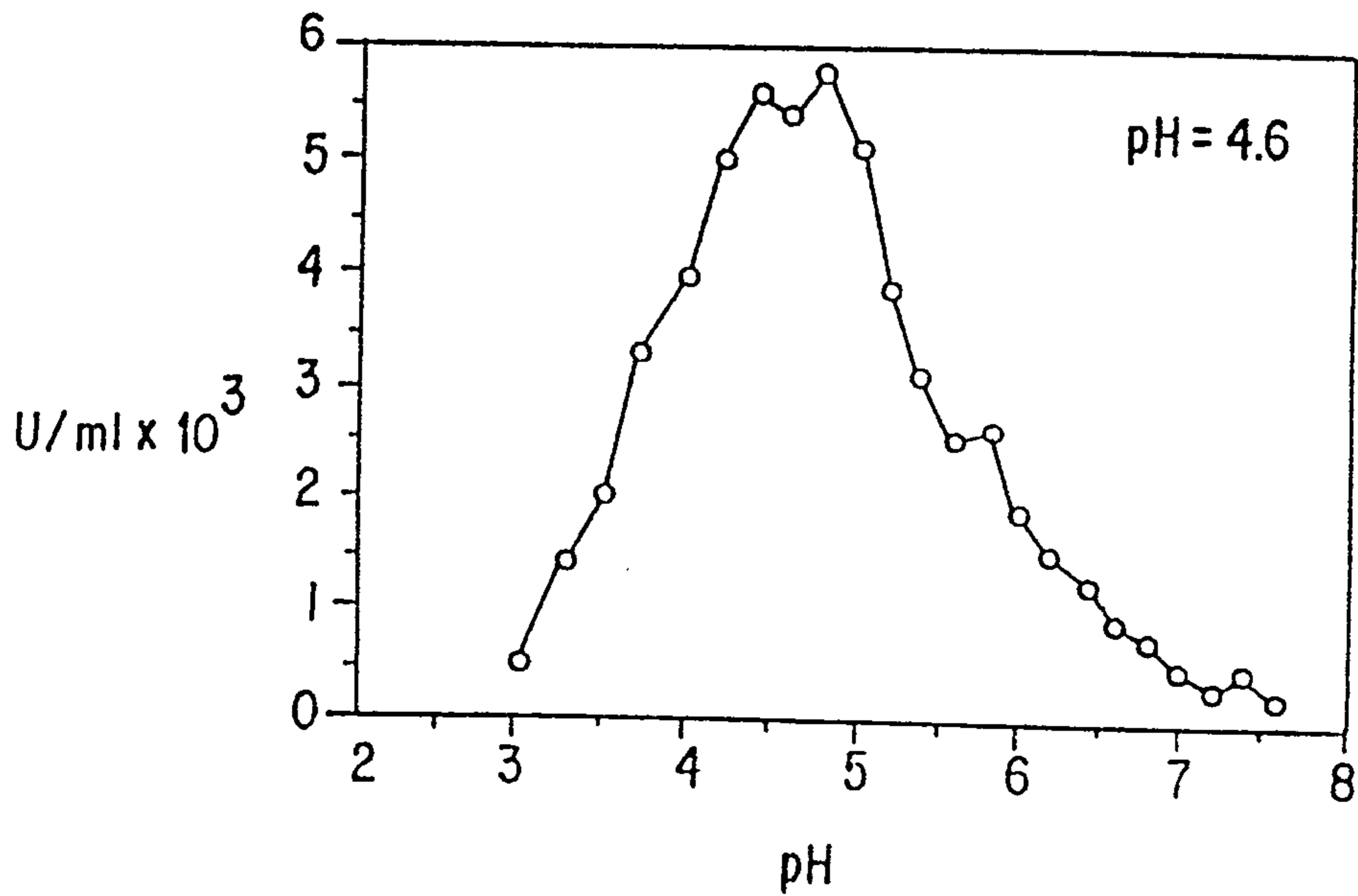
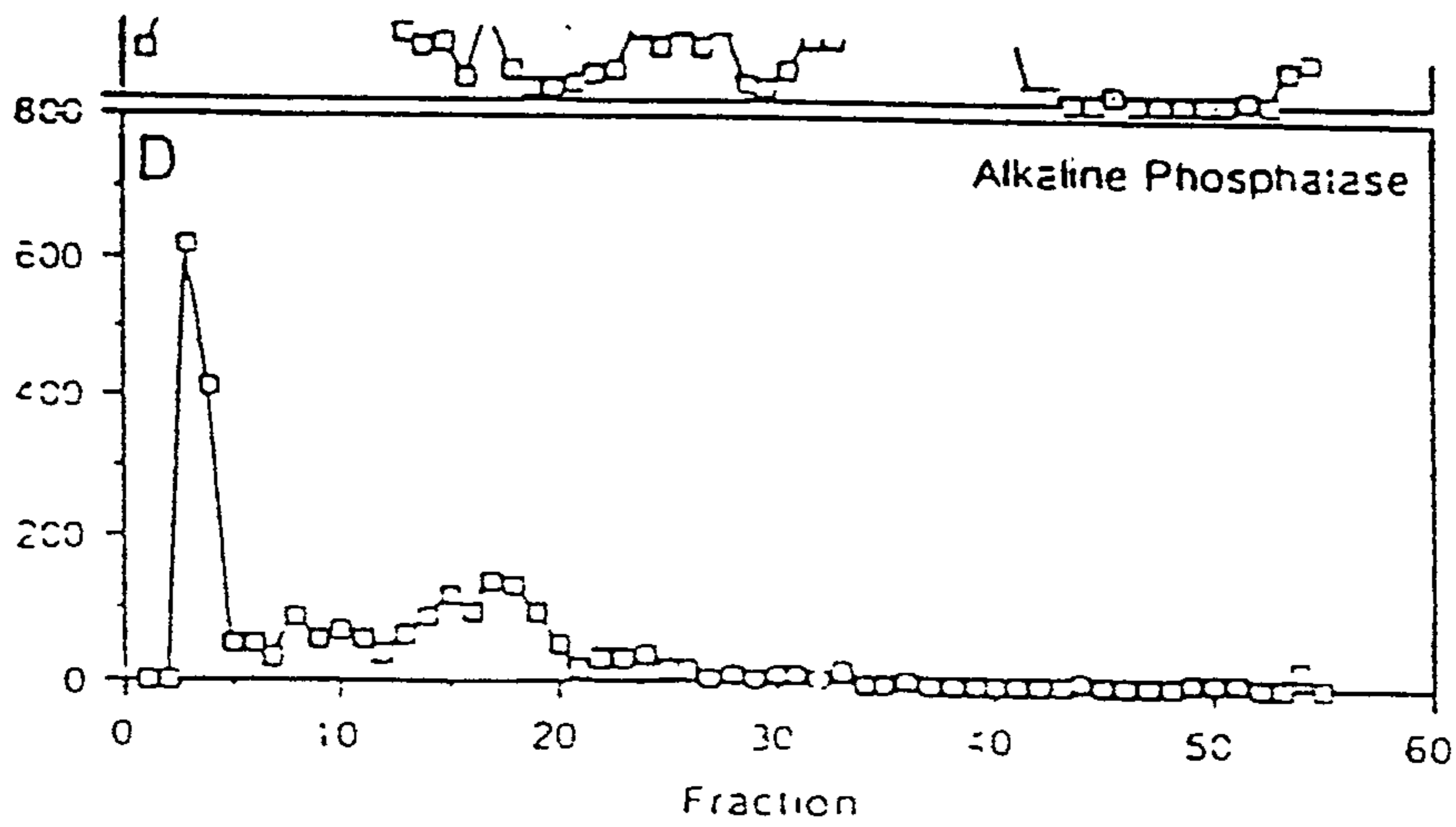
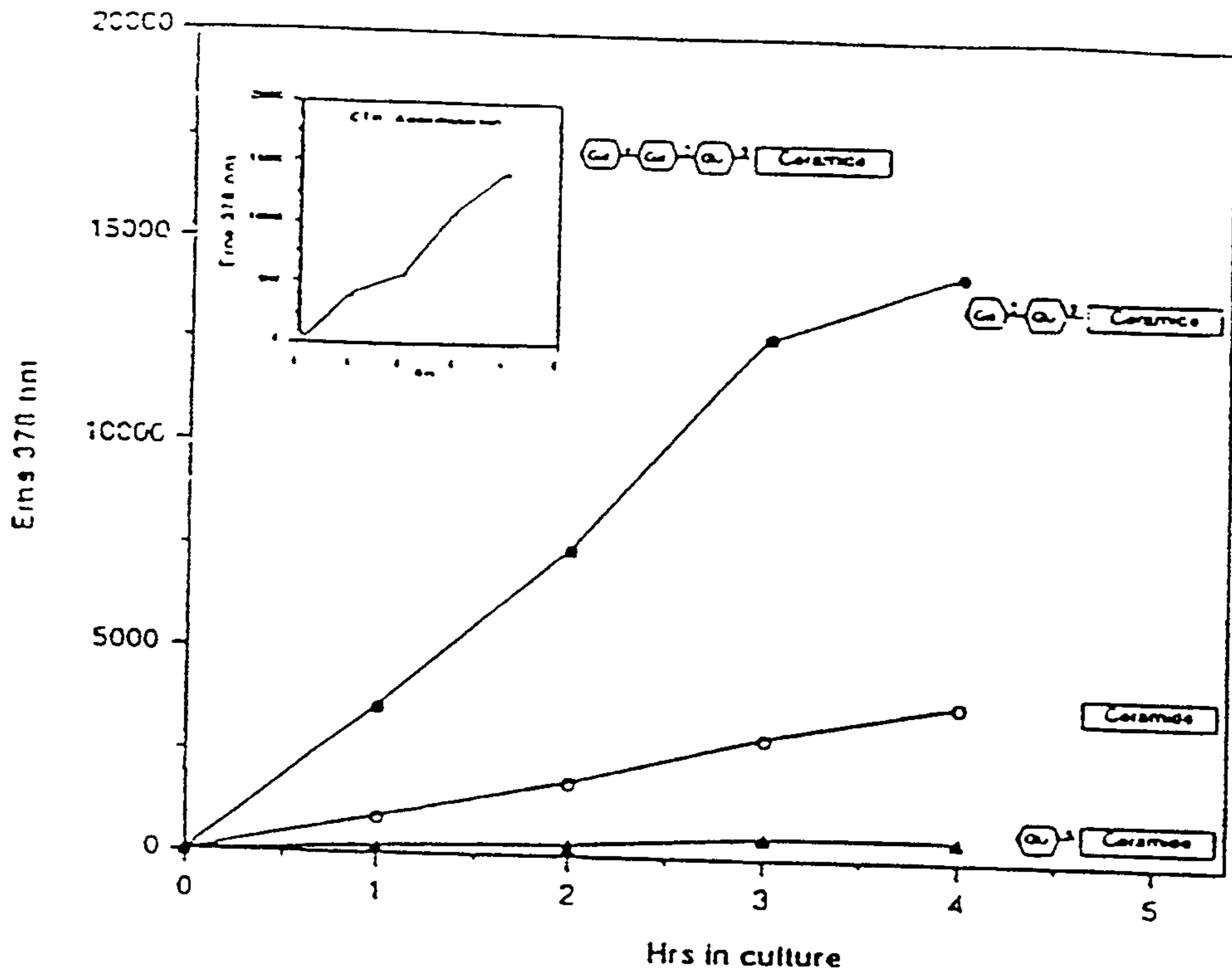


FIG.7C

FIG. 8



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kDa

92 —

57 —

36 —

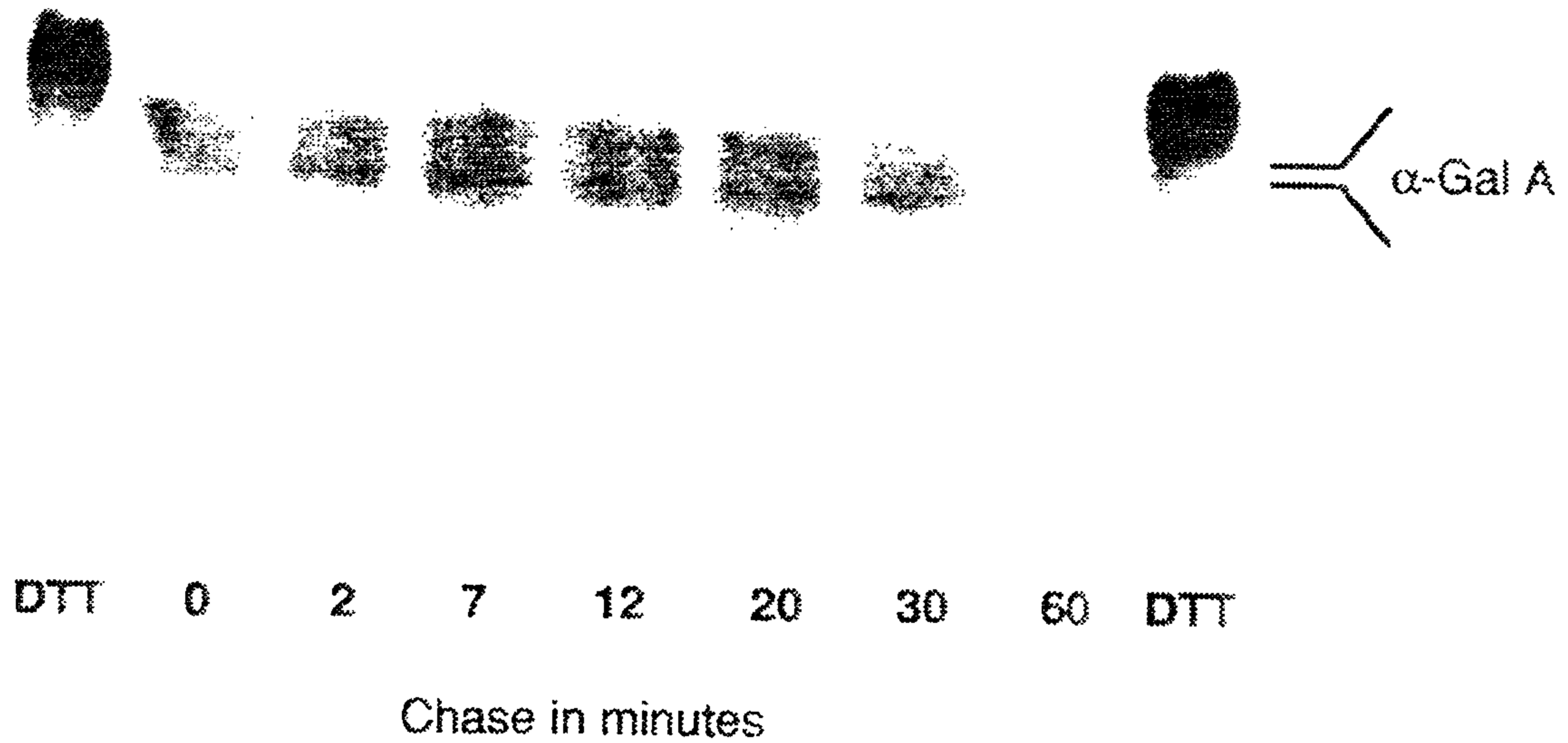


FIG. 9

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kDa

92 —

57 —

36 —

0 2 7 12 20 30 60

CHASE IN MINUTES

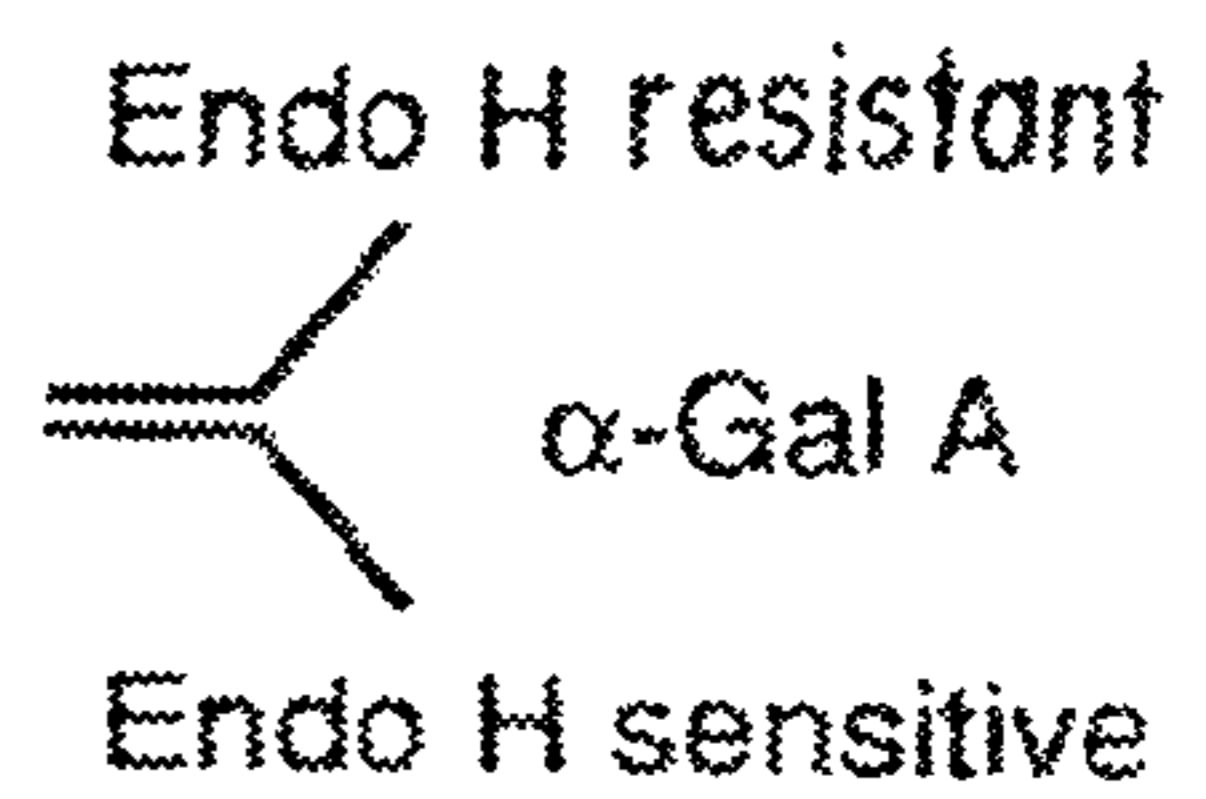


FIG. 10

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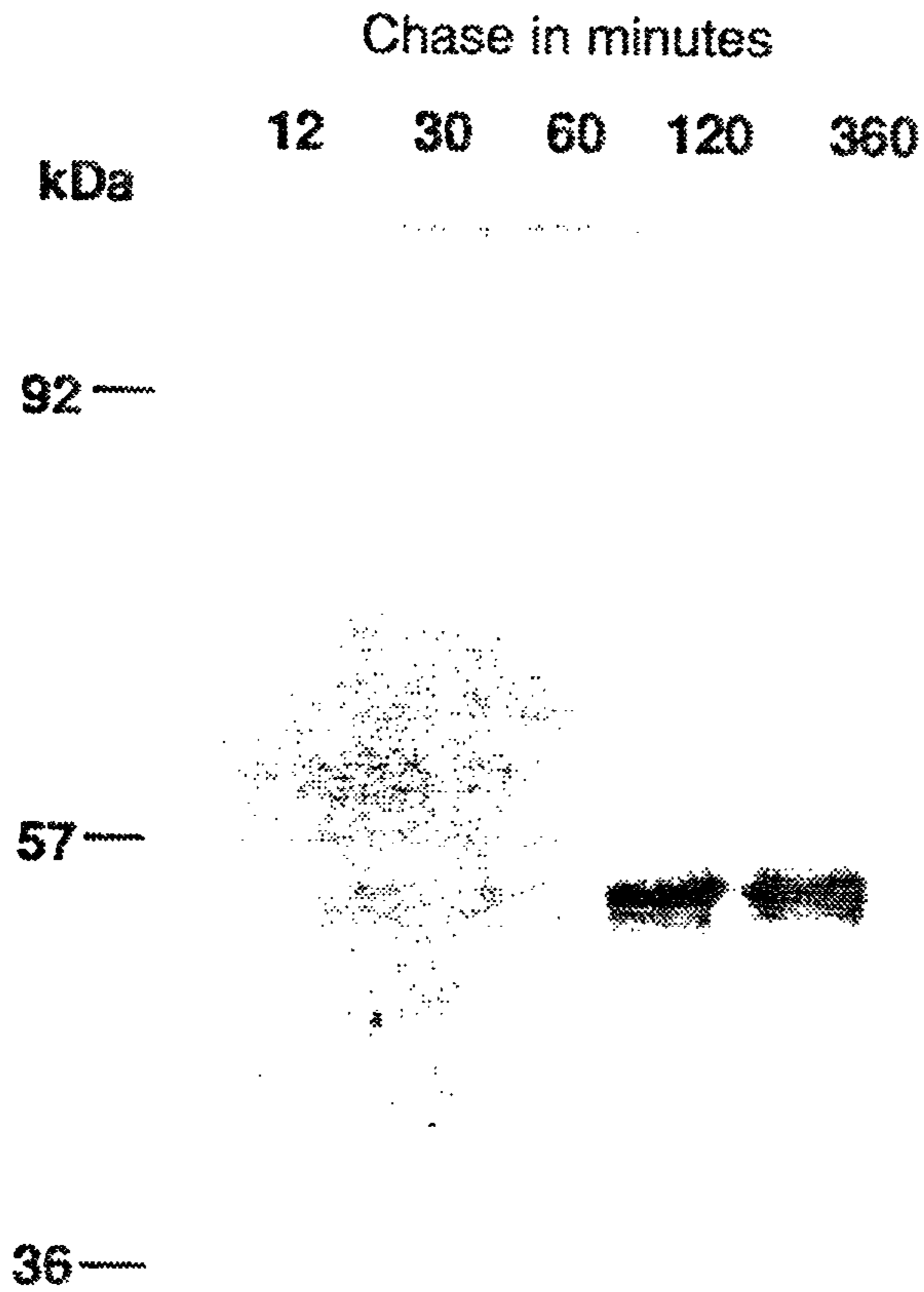


FIG. 11

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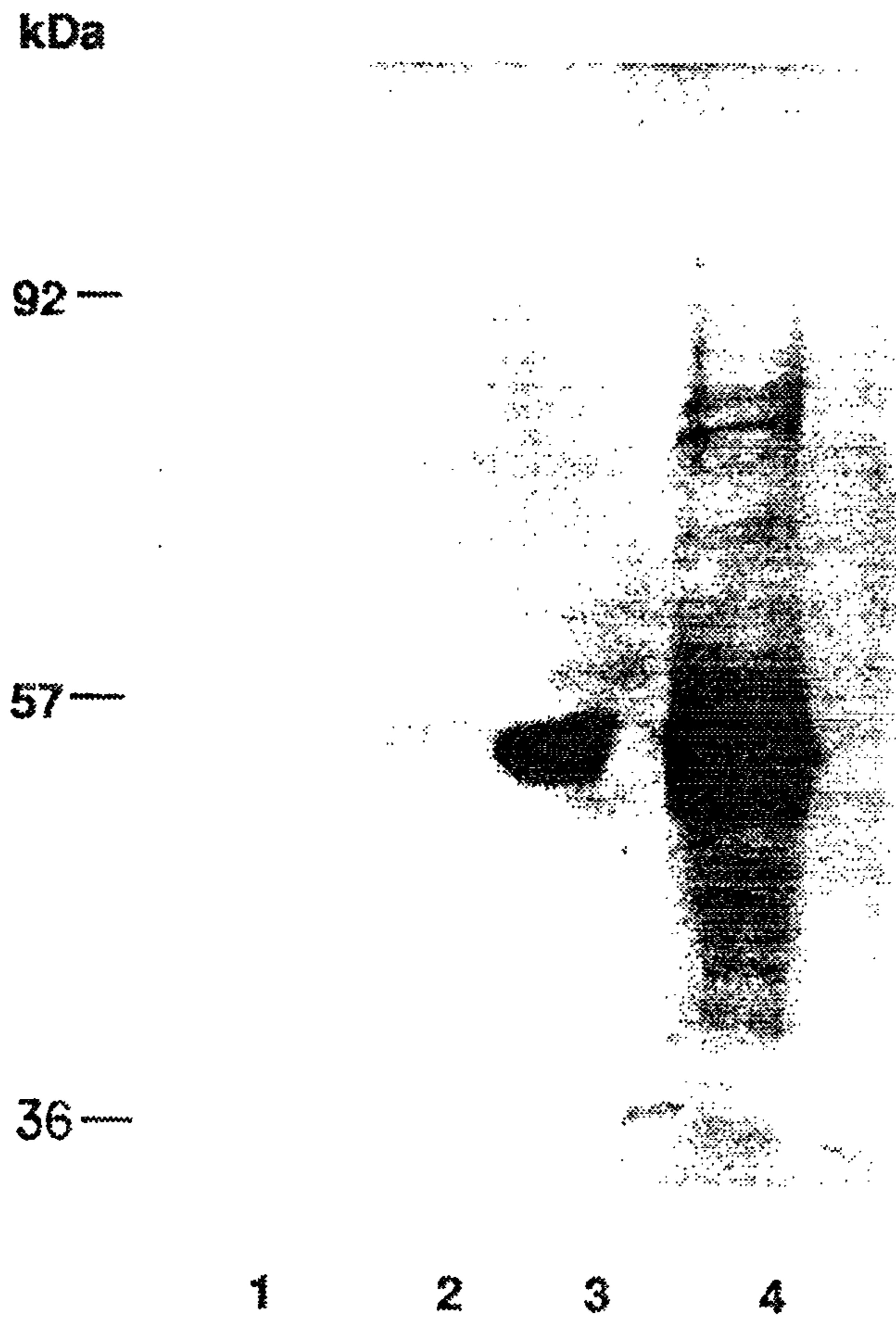


FIG. 12

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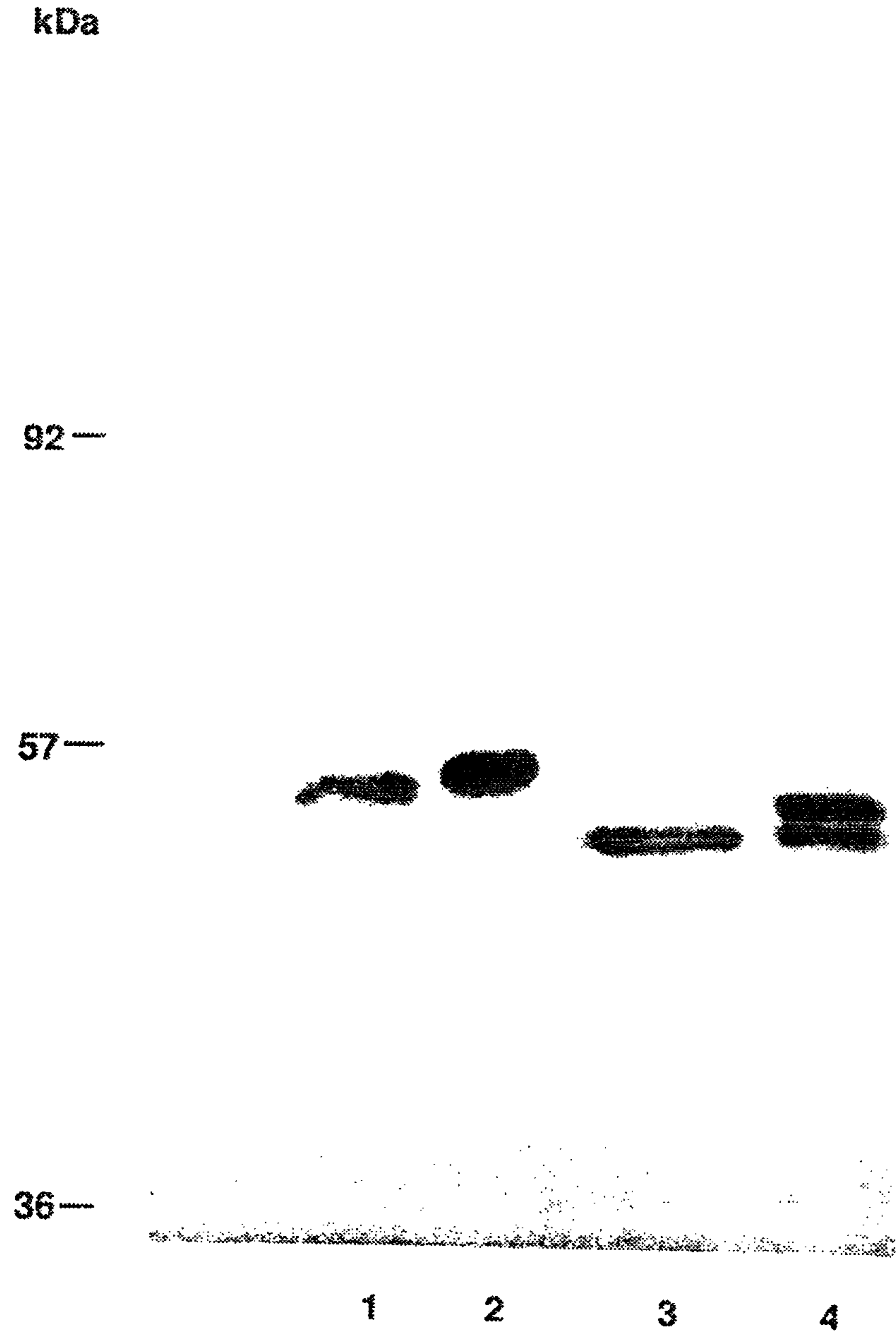


FIG. 14

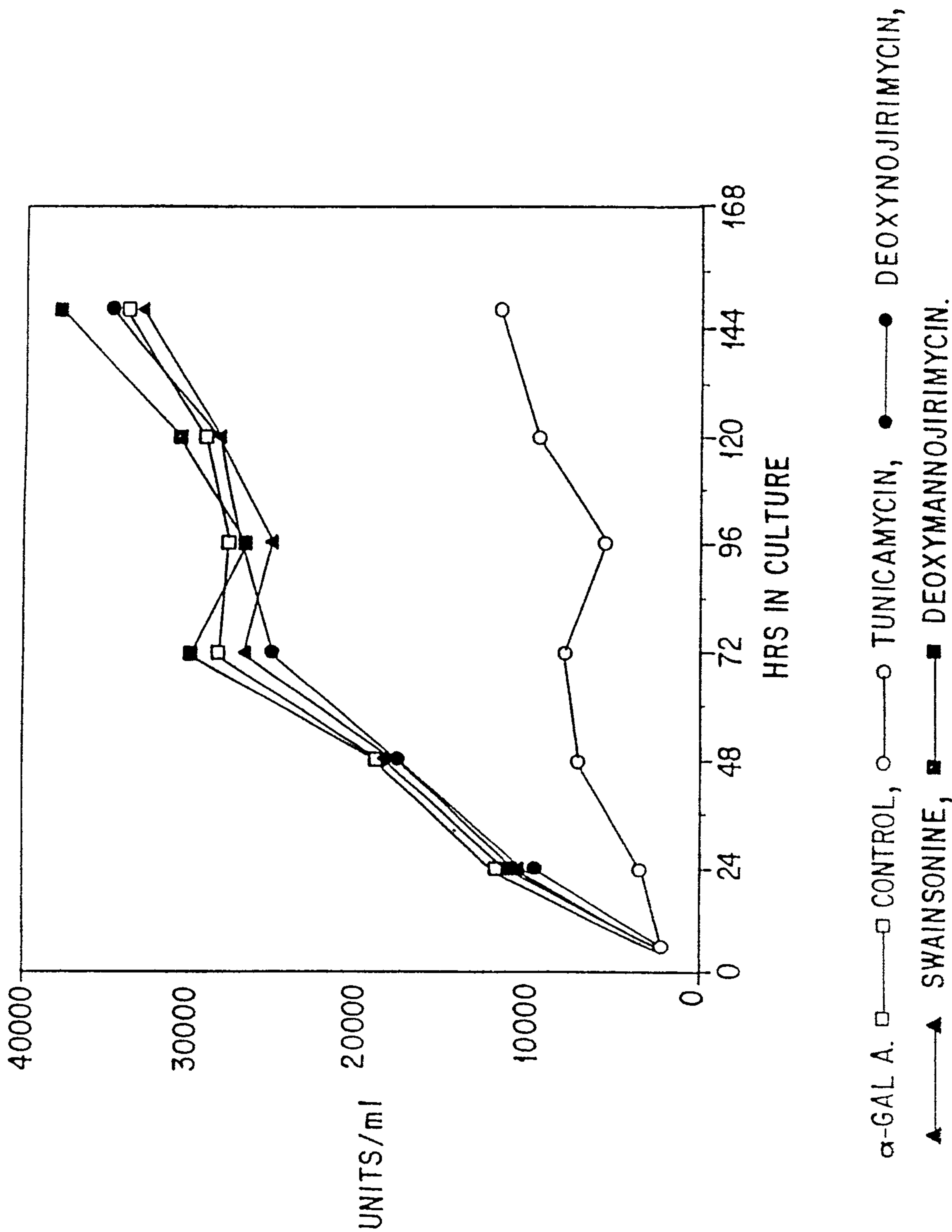


FIG.15

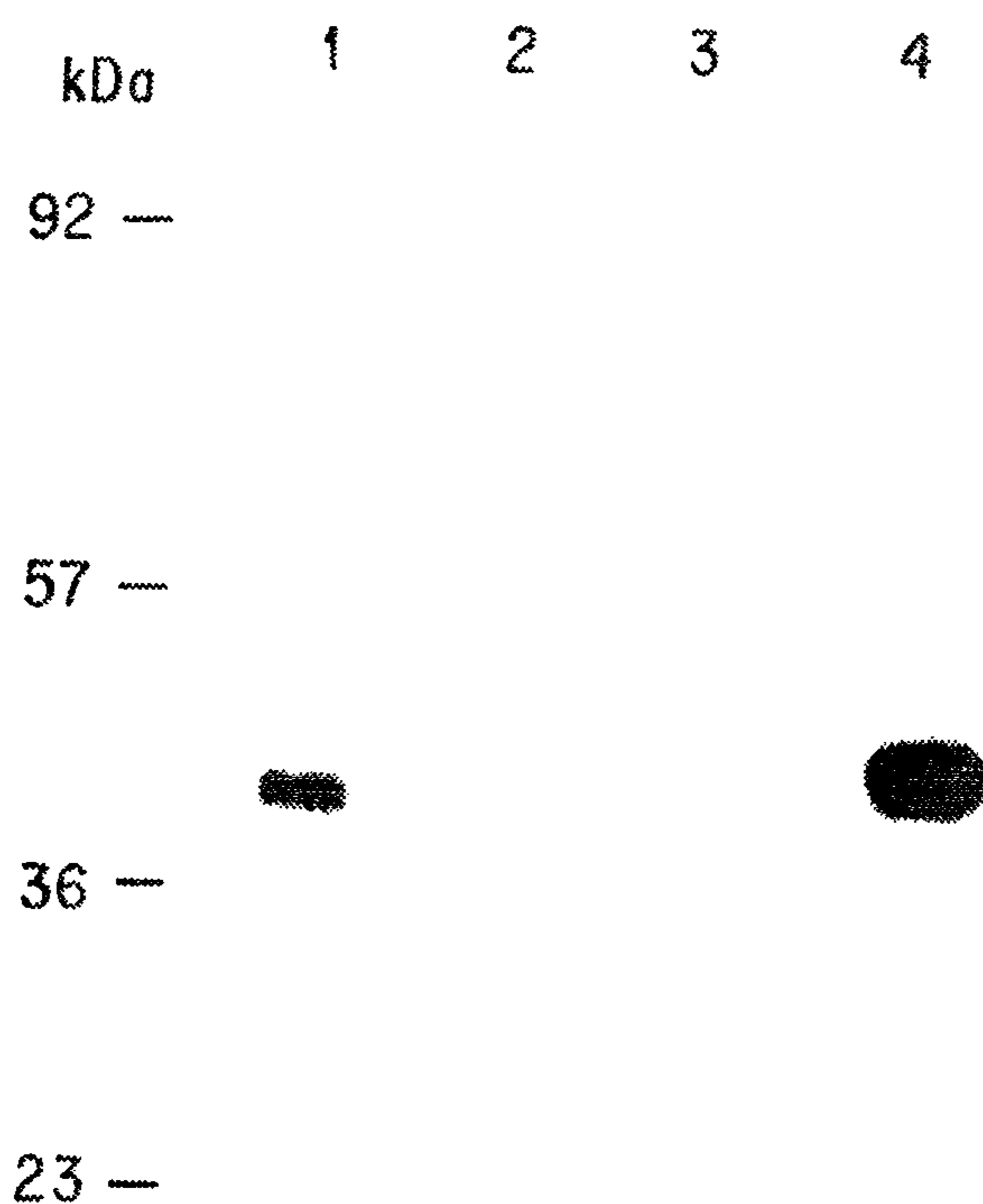


FIG. 16

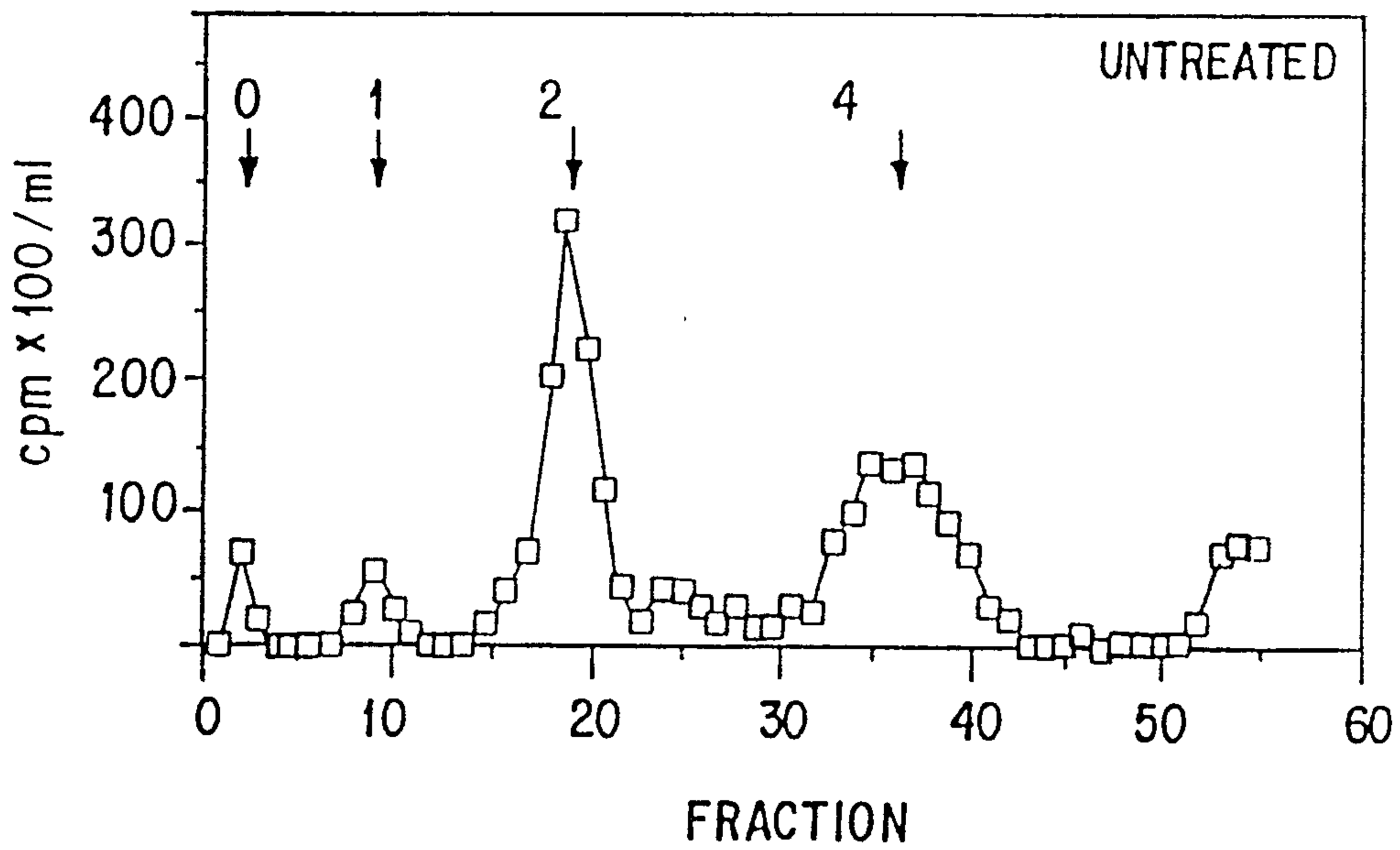


FIG. 17A

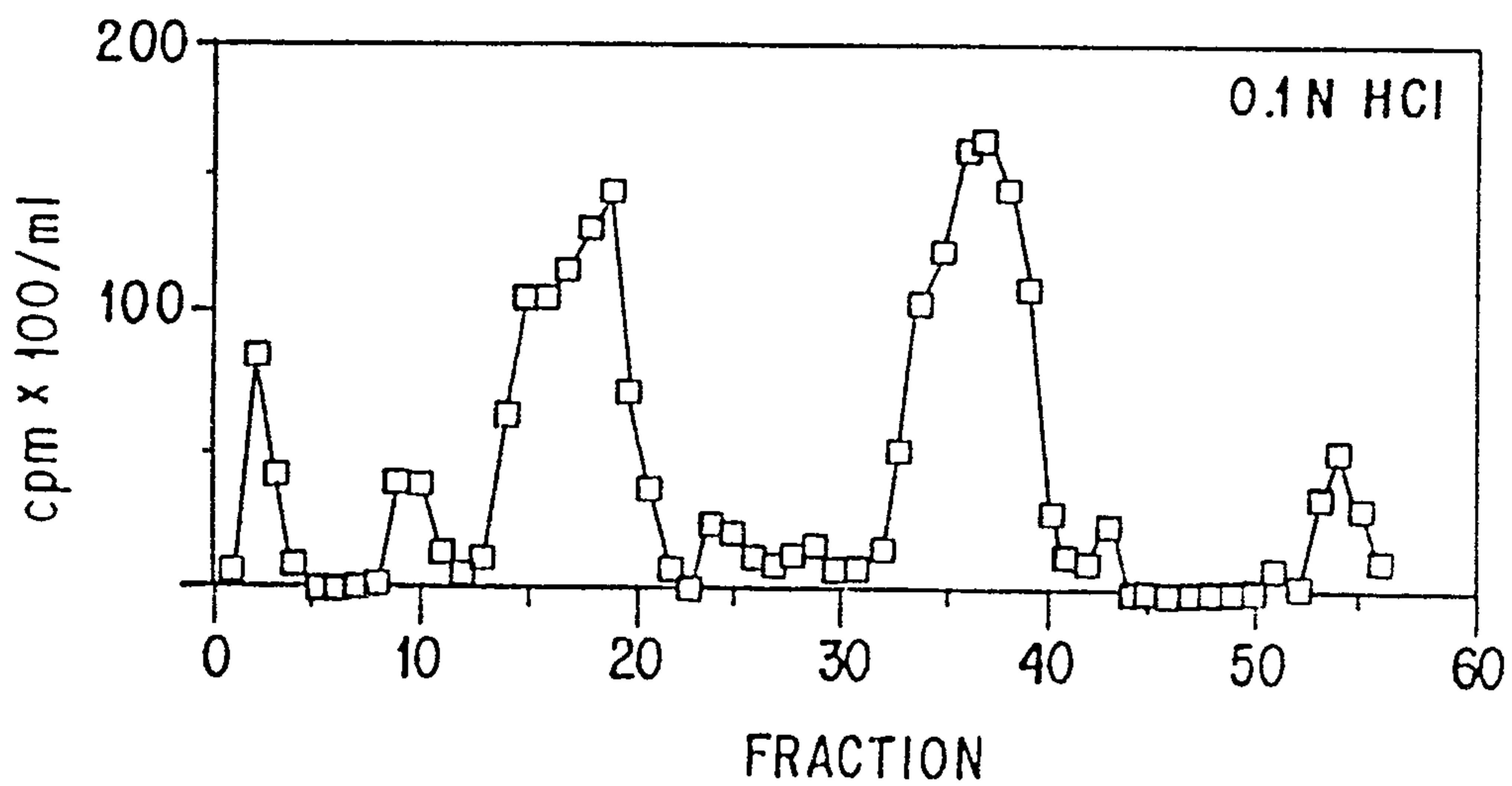


FIG. 17B

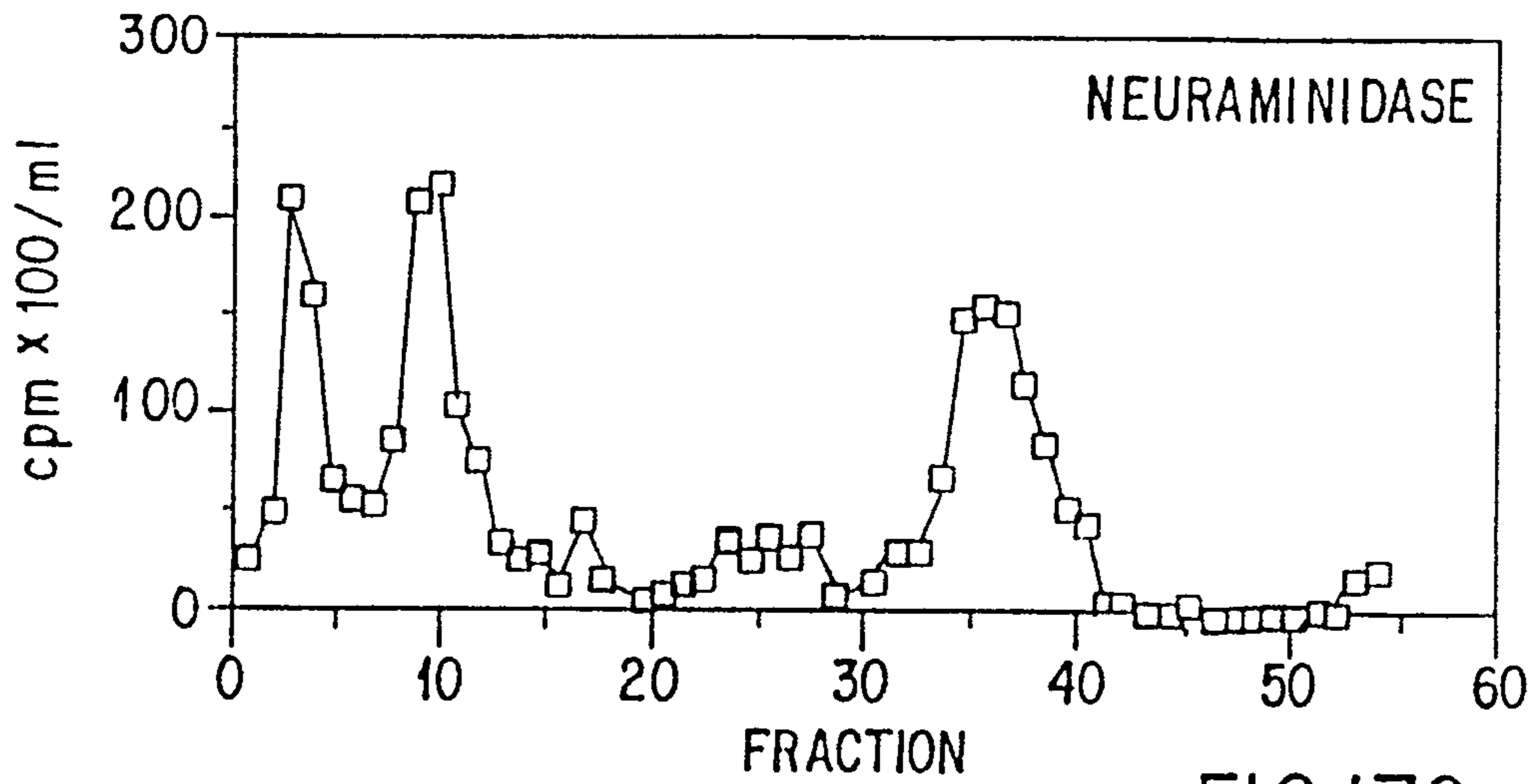


FIG.17C

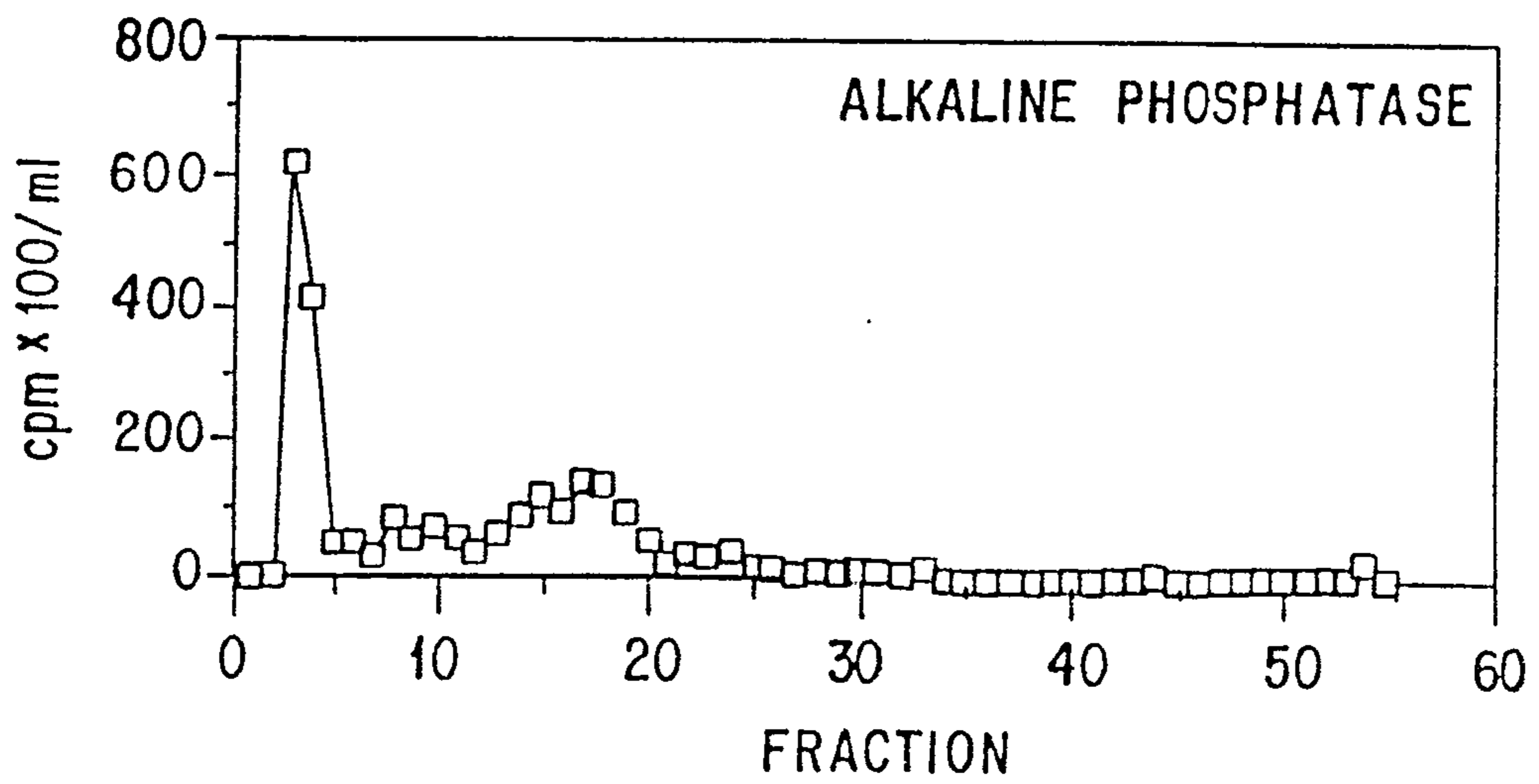


FIG.17D

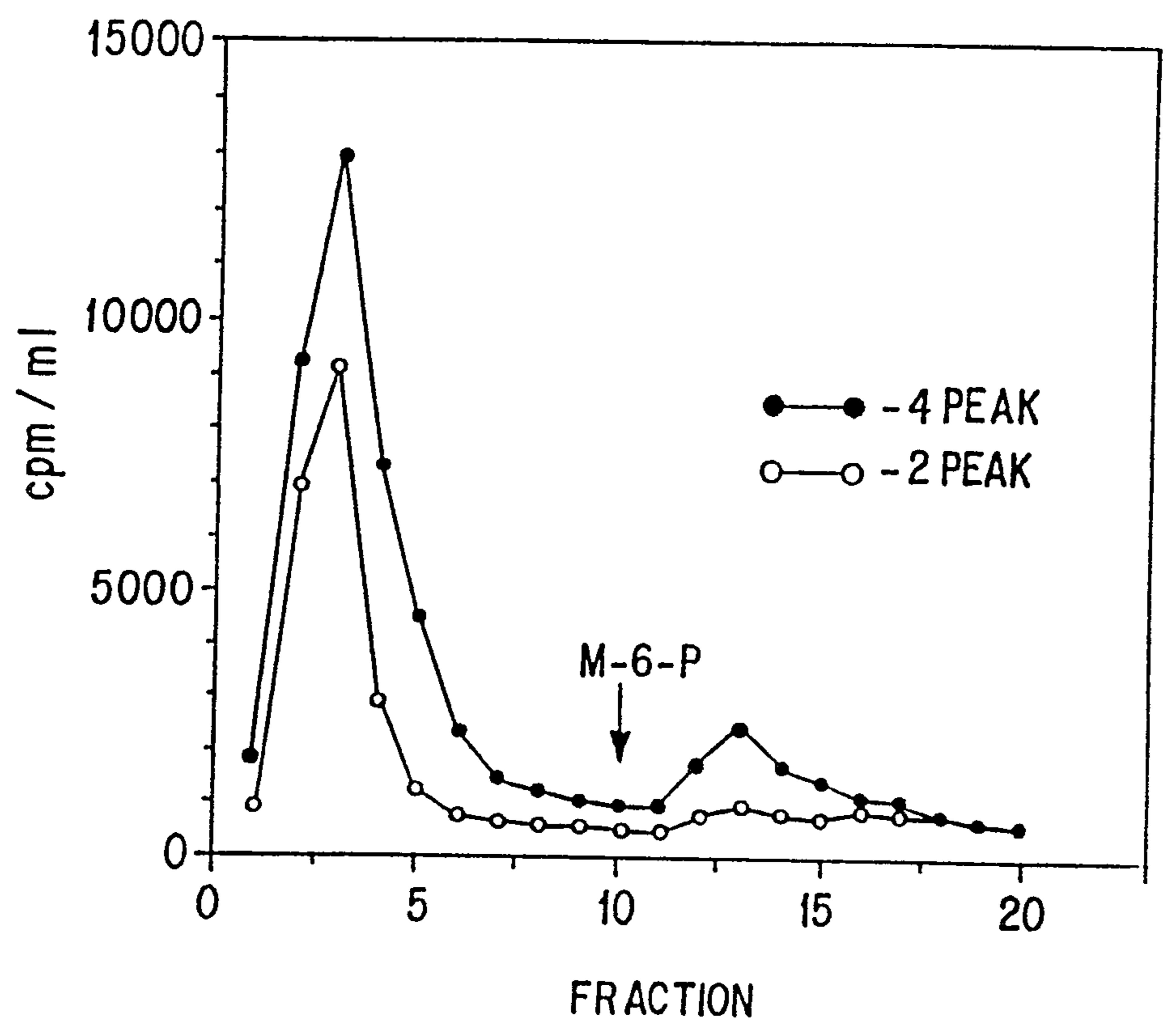


FIG.18

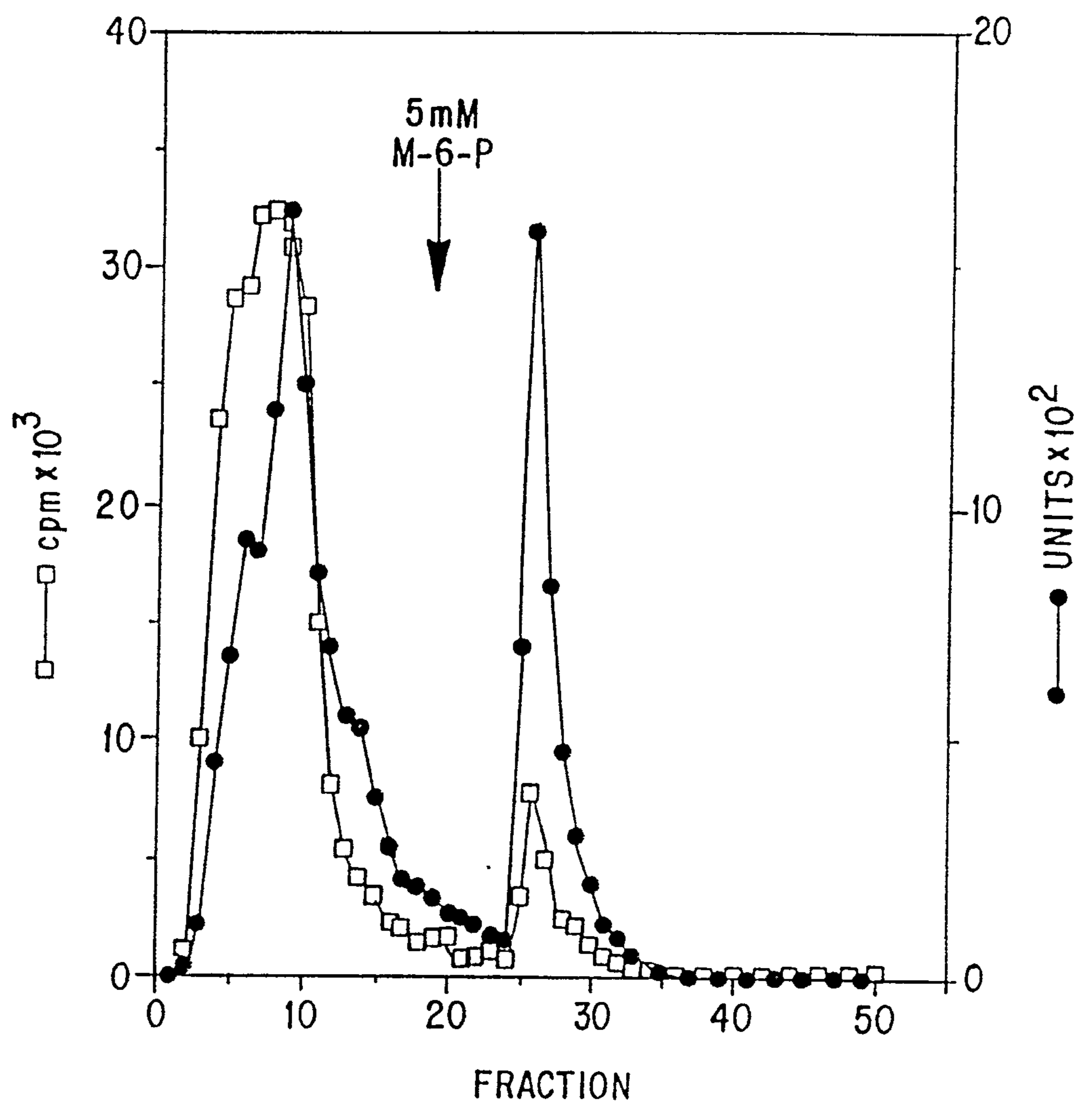


FIG. 19

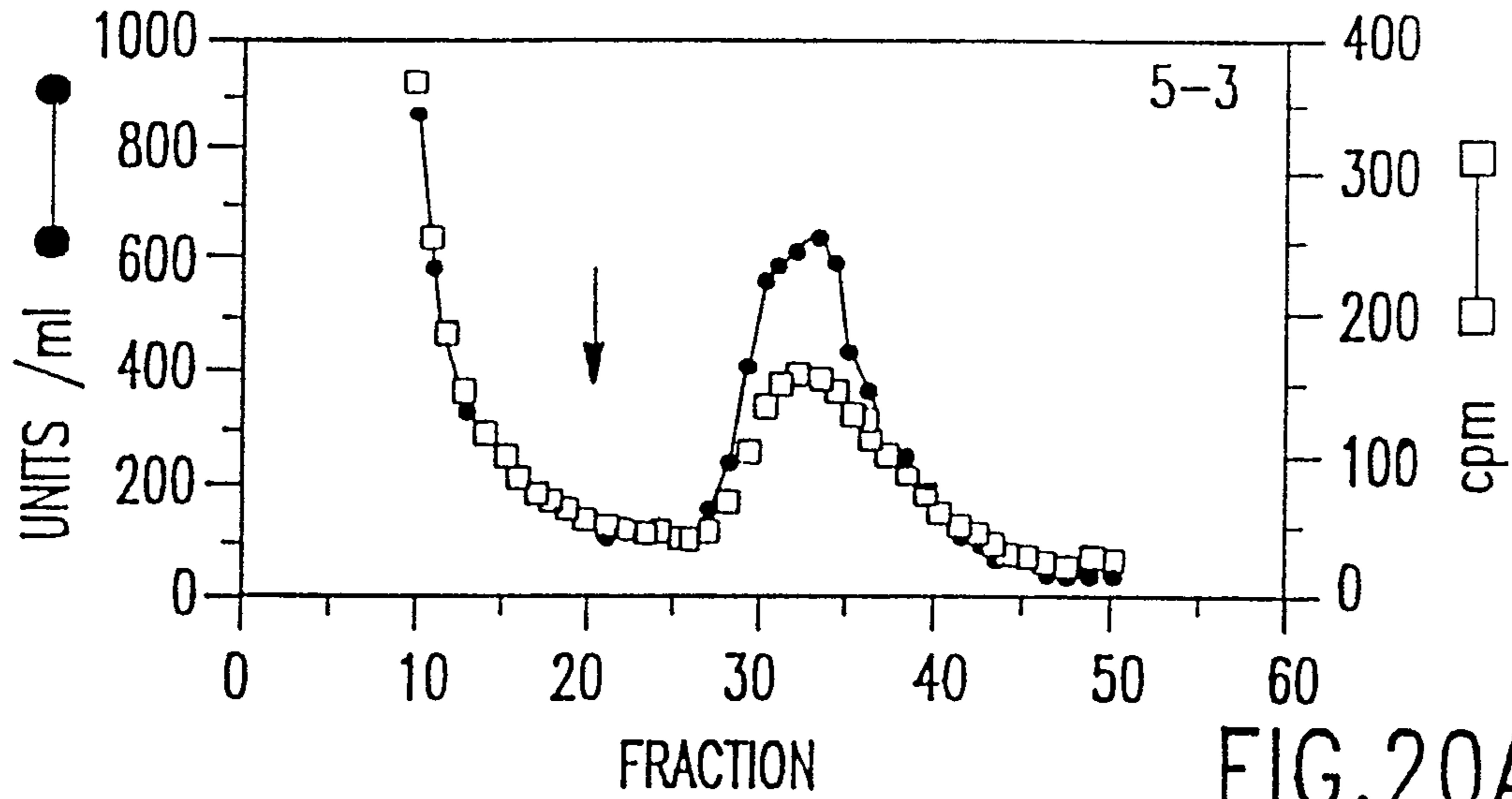


FIG.20A

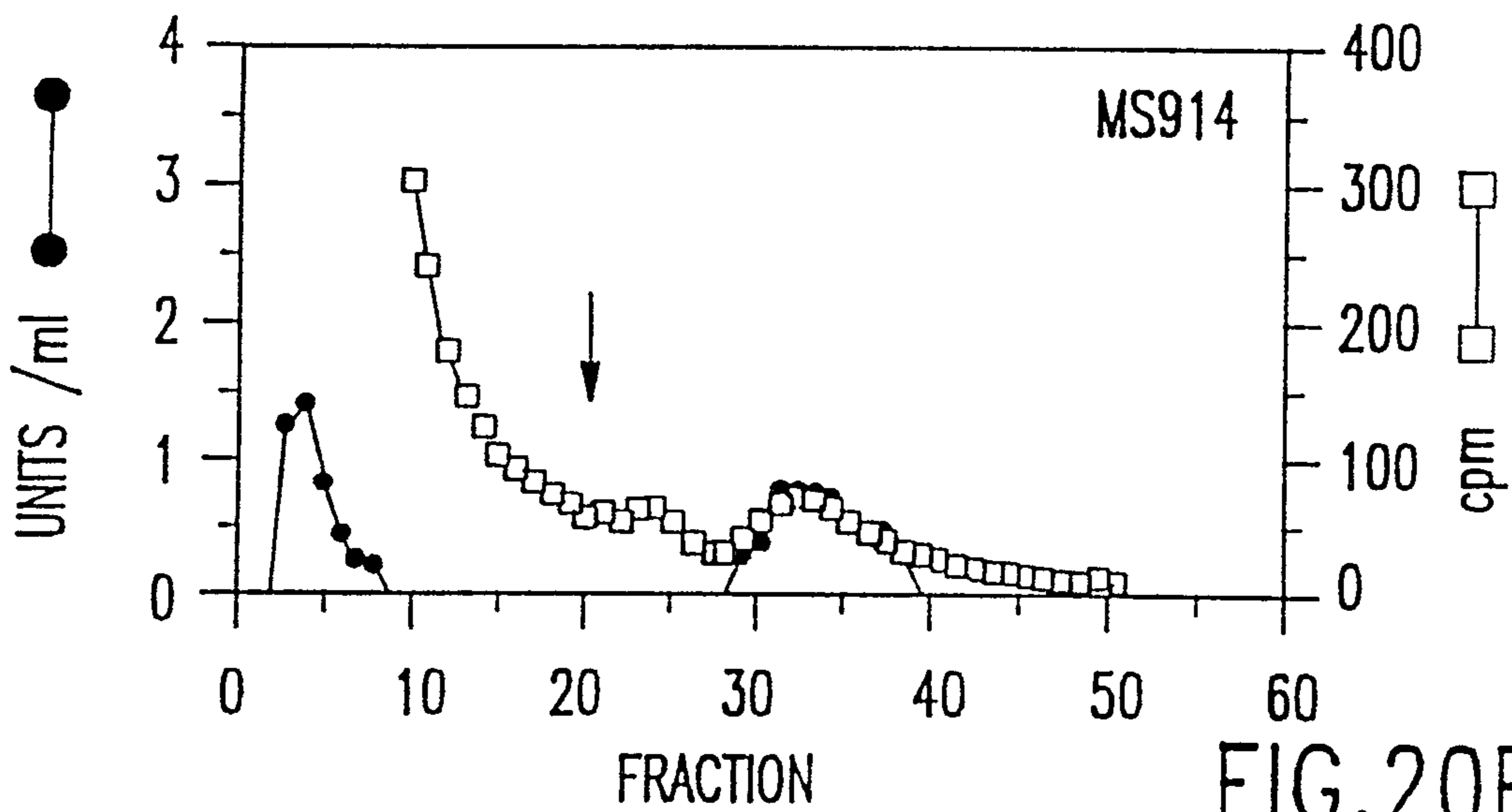


FIG.20B

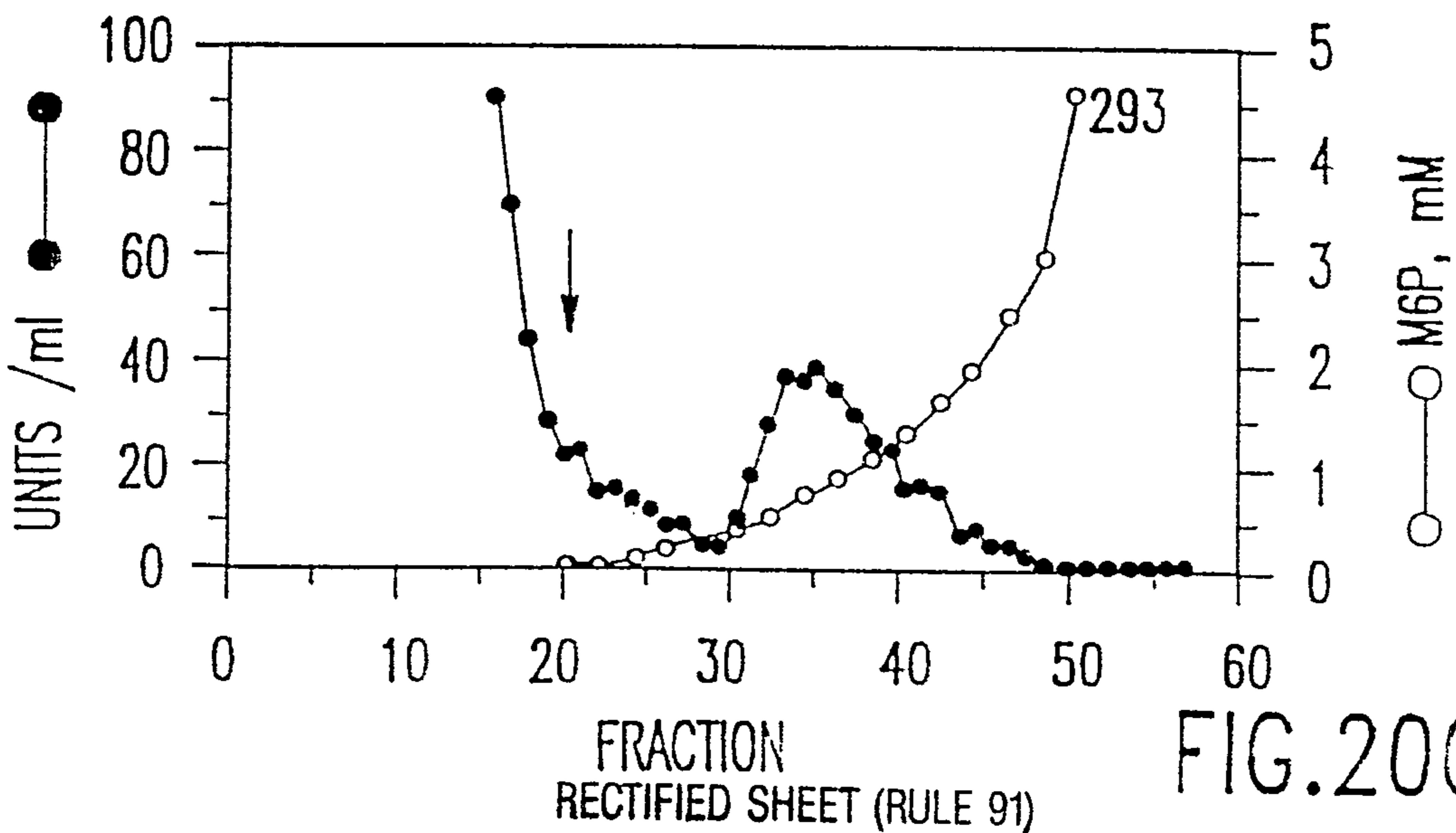


FIG.20C

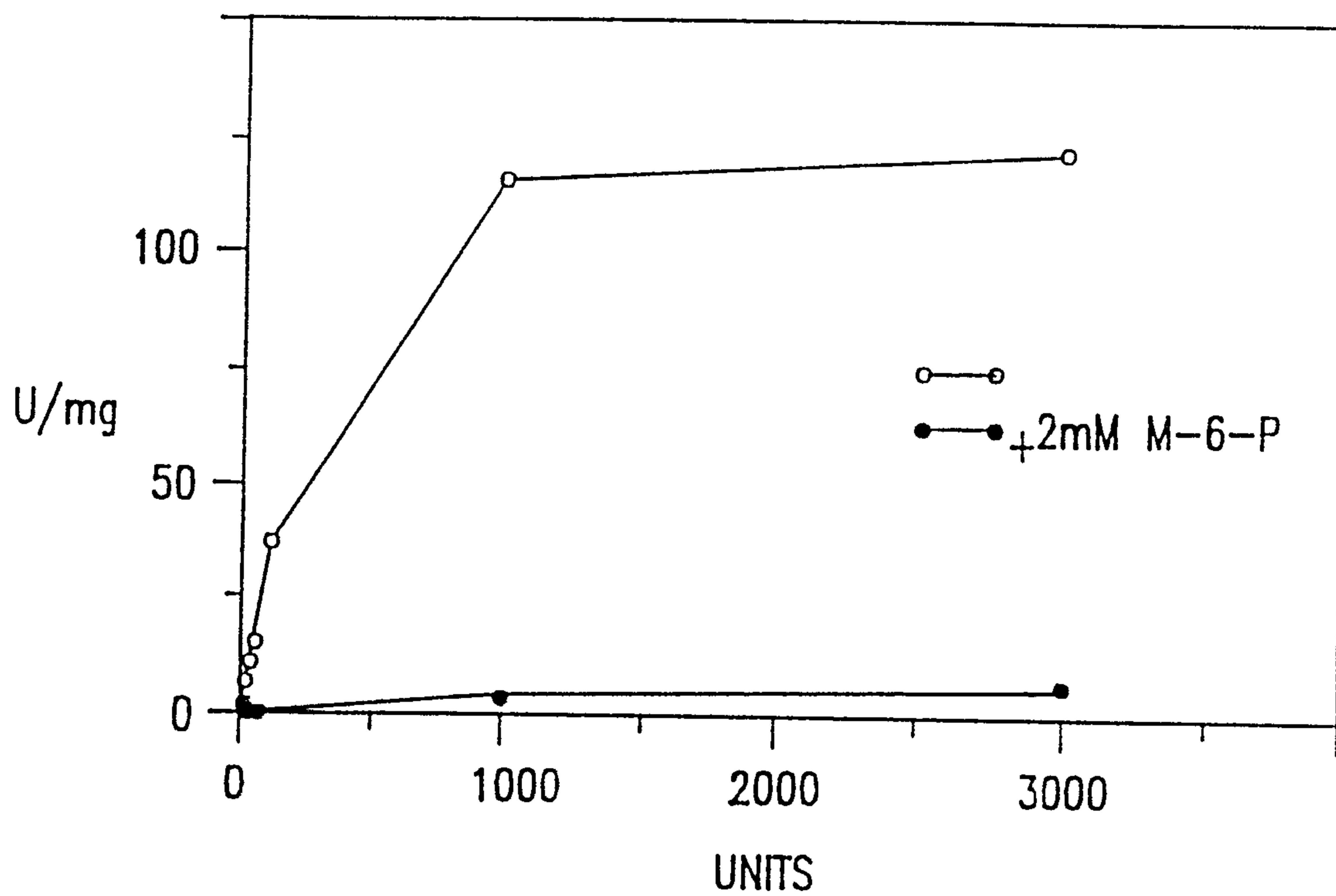


FIG.21

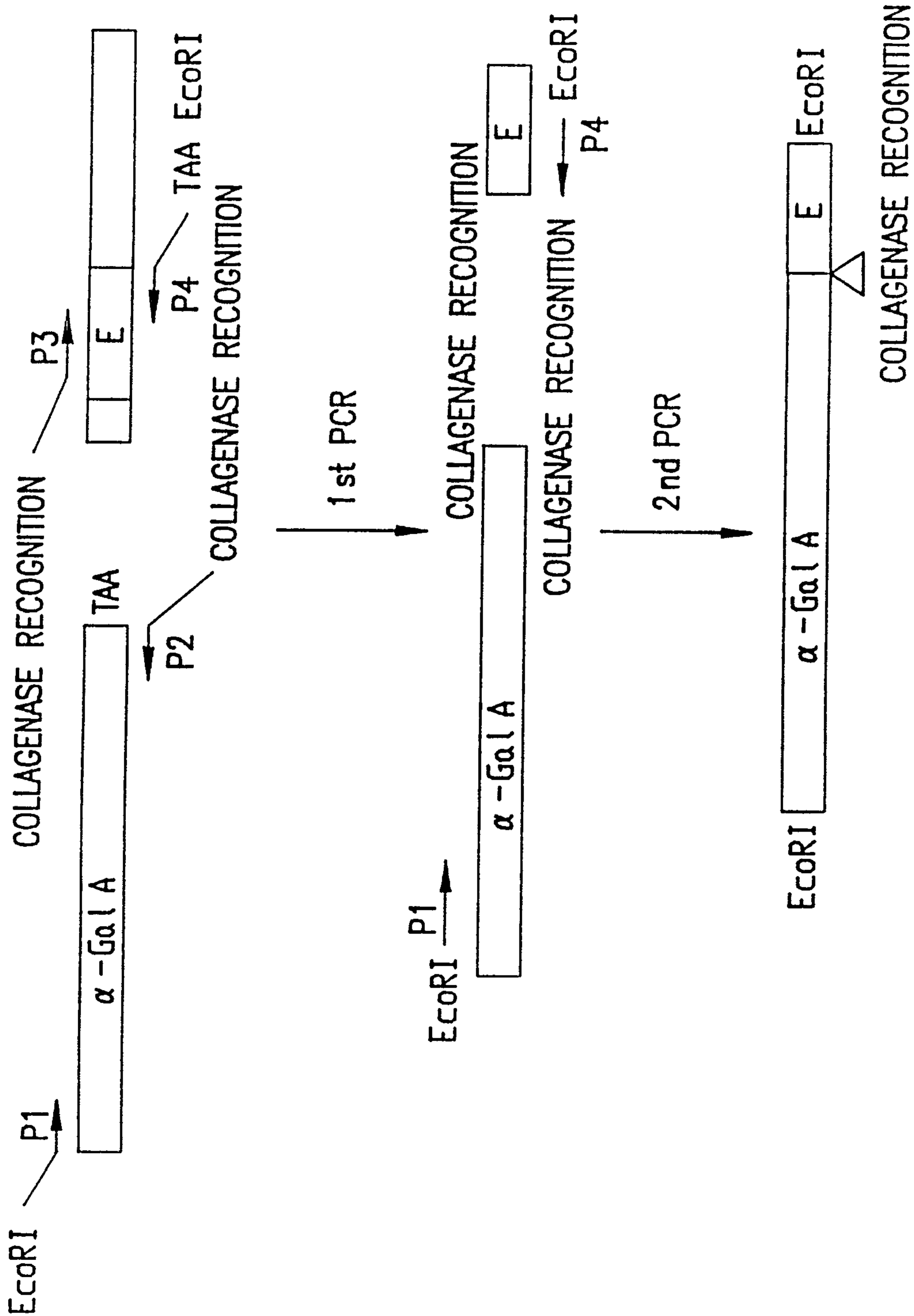


FIG.22

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1191 GAA TGG ACT TCA AGG TTA AGA AGT CAC ATA AAT CCC ACA GGA ACT
 398 Glu Trp Thr Ser Arg Leu Arg Ser His Ile Asn Pro Thr Gly Thr

1226 GTT TTG CTT CAG CTA GAA AAT ACA ATG CAG ATG TCA TTA AAA GAC
 413 Val Leu Leu Gln Leu Glu Asn Thr Met Gln Met Ser Leu Lys Asp

Collagenase cleavage

1271 TTA CTT CCG GCT GGT CCG GCG CAA CAC GAT GAA GCT CAA CAA AAT
 428 Leu Leu Pro Ala Gly Pro Ala Gln His Asp Glu Ala Gln Gln Asn

α -Gal A

IqG Binding domain E

1316 GCT TTT TAT CAA GTC TTA AAT ATG CCT AAC TTA AAT GCT GAT CAA
 443 Ala Phe Tyr Gln Val Leu Asn Met Pro Asn Leu Asn Ala Asp Gln

1371 CGC AAT GGT TTT ATC CAA AGC CTT AAA GAT GAT CCA AGC CAA AGT
 458 Arg Asn Gly Phe Ile Gln Ser Leu Lys Asp Asp Pro Ser Gln Ser

1416 GCT AAC GTT TTA GGT GAA GCT CAA AAA CTT AAT GAC TCT CAA GCT
 473 Ala Asn Val Leu Gly Glu Ala Gln Lys Leu Asn Asp Ser Gln Ala

1501 CCA AAA TAA Bam HI Eco RI
 488 Pro Lys Ter GGATCCCGGAATTCGGCC

FIG.23

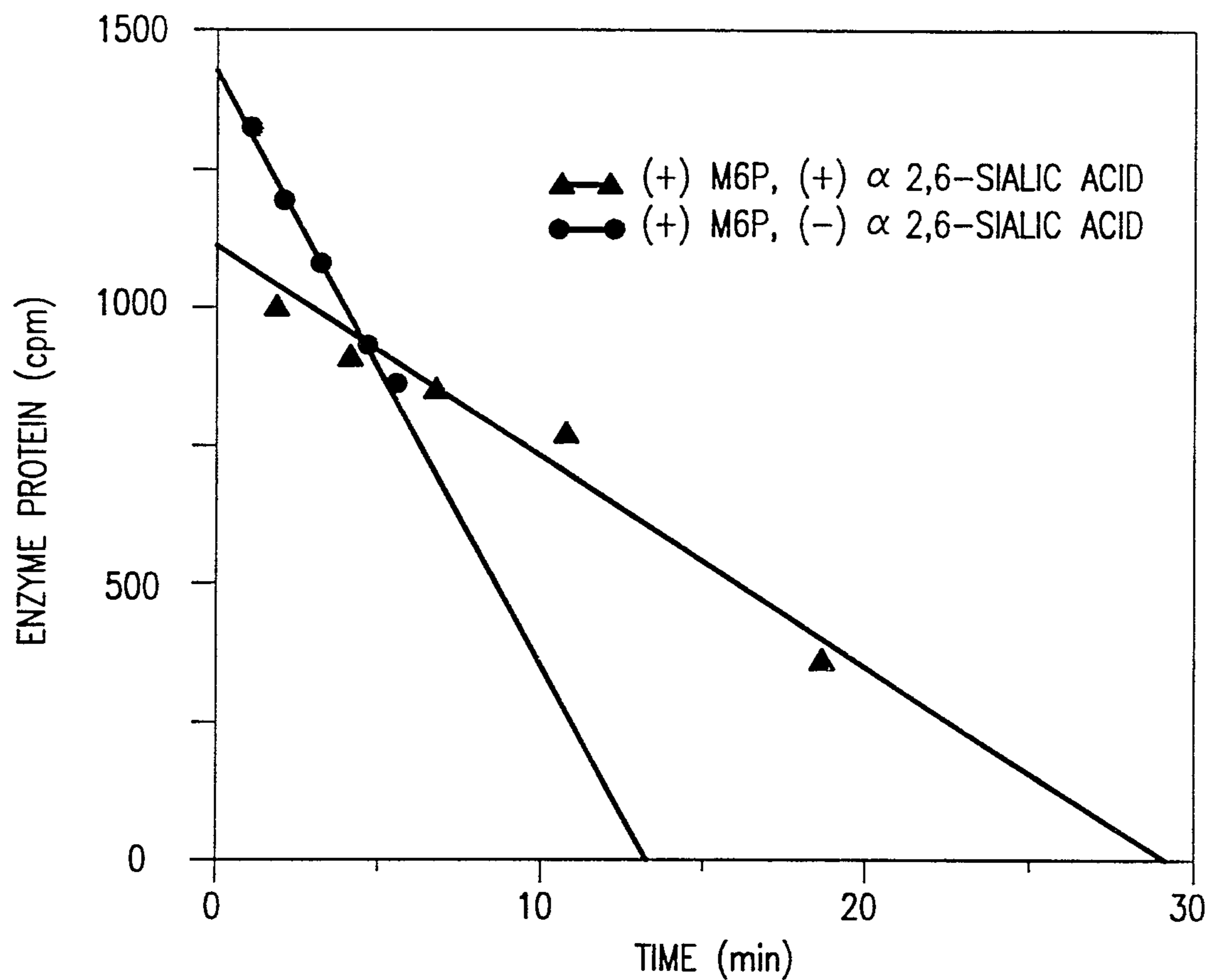


FIG.24A

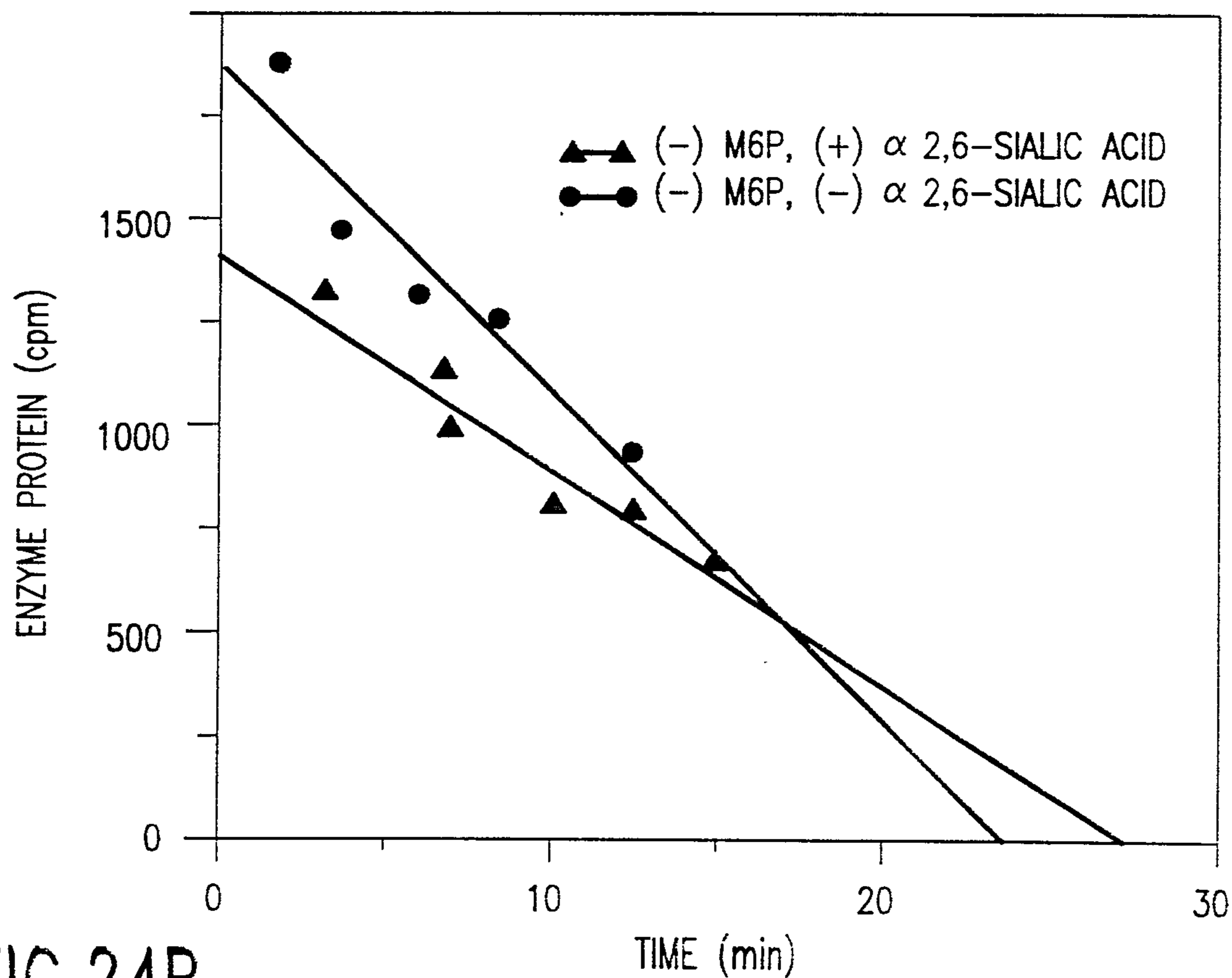
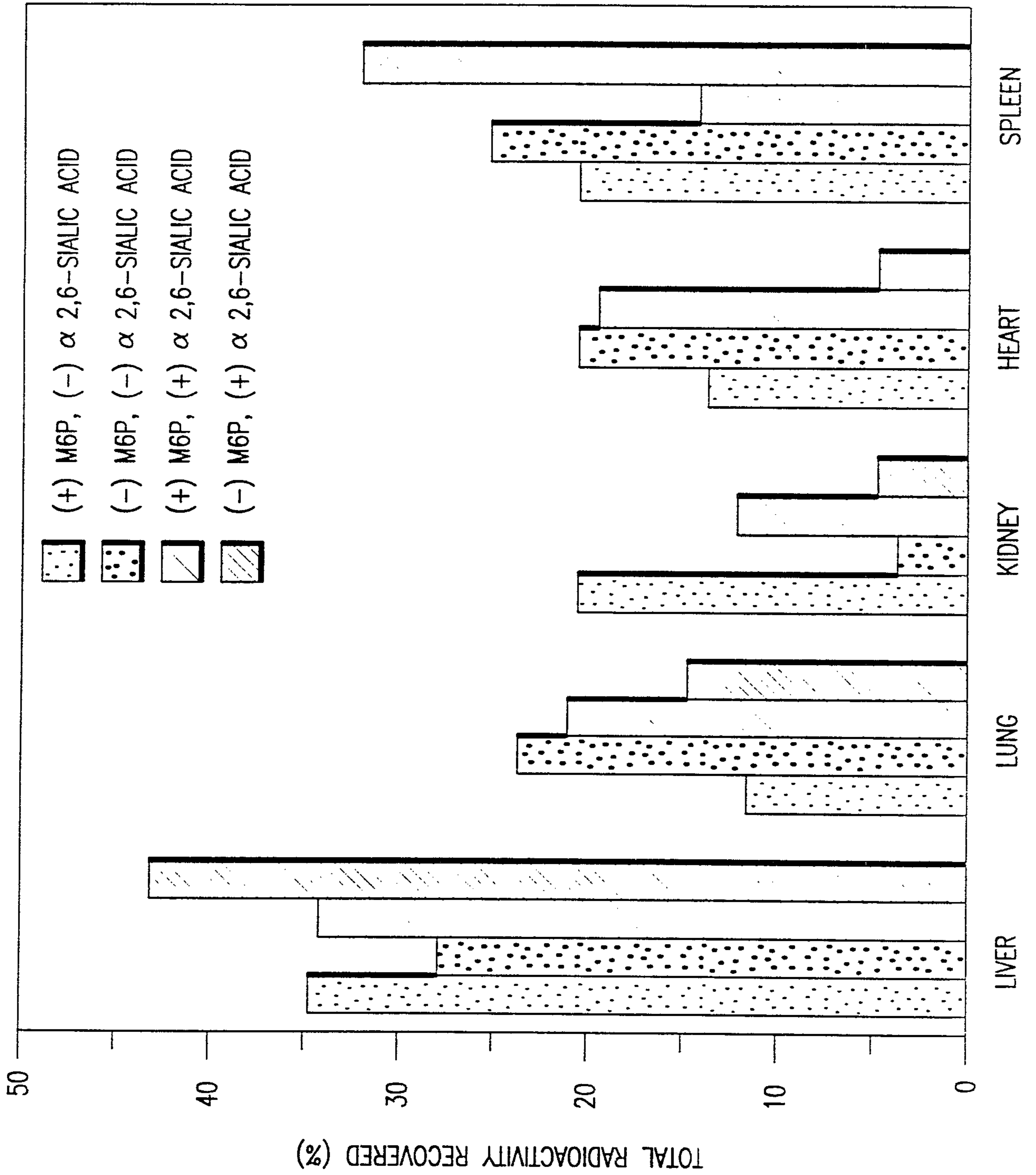


FIG.24B



RECTIFIED SHEET (RULE 91)

FIG.25

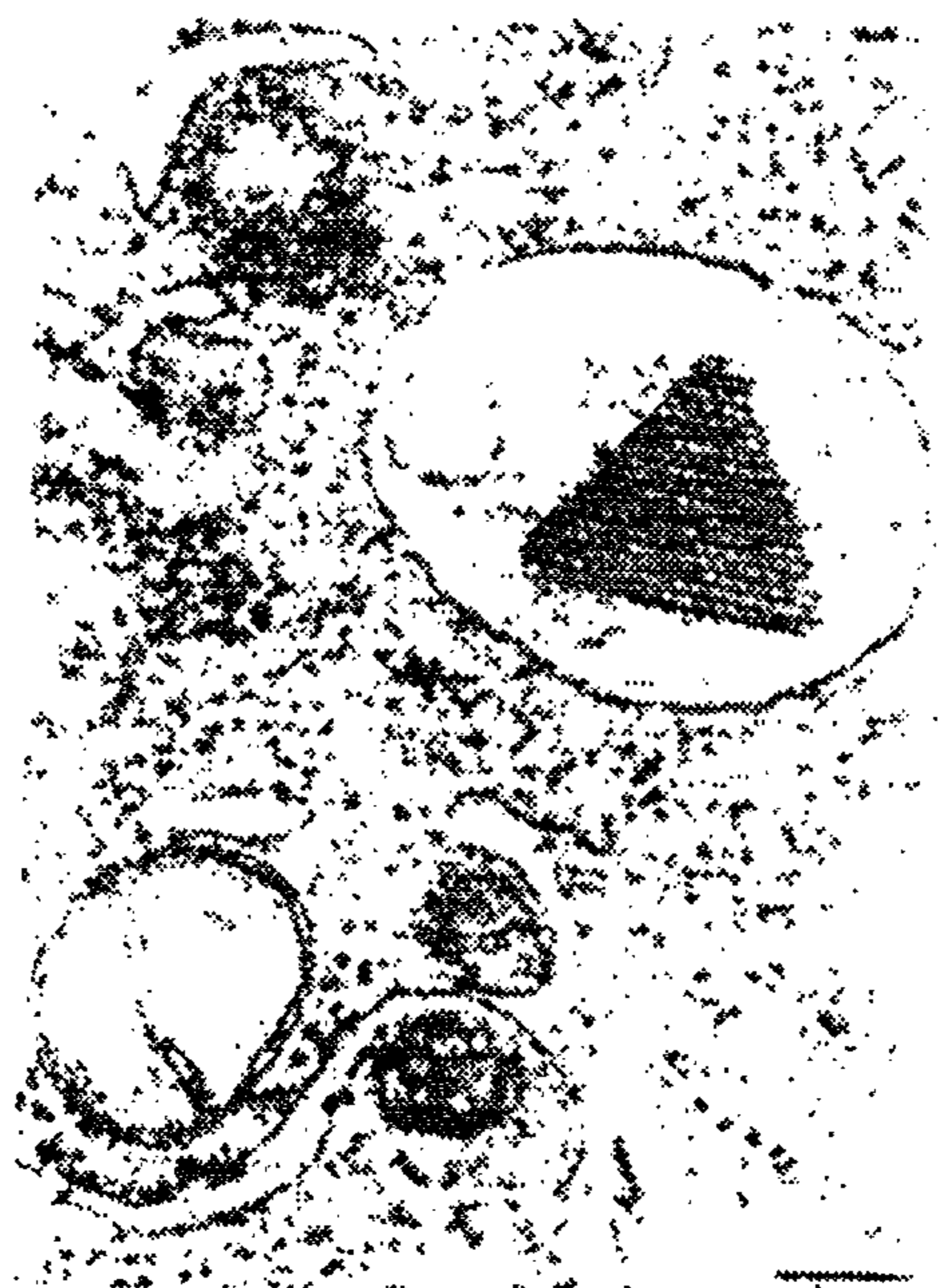


FIG. 26A



FIG. 26B



FIG. 26C



FIG. 26D

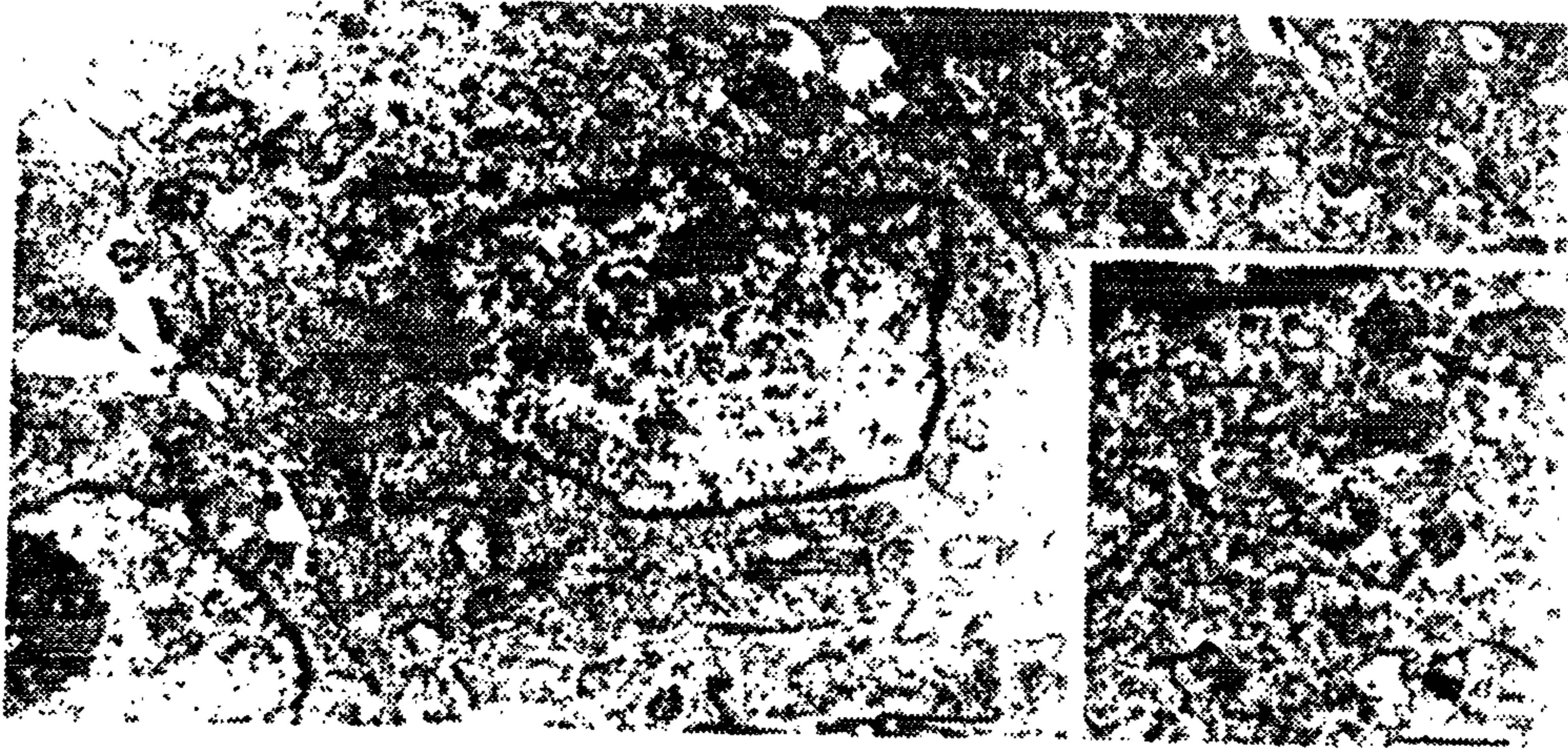


FIG. 26E

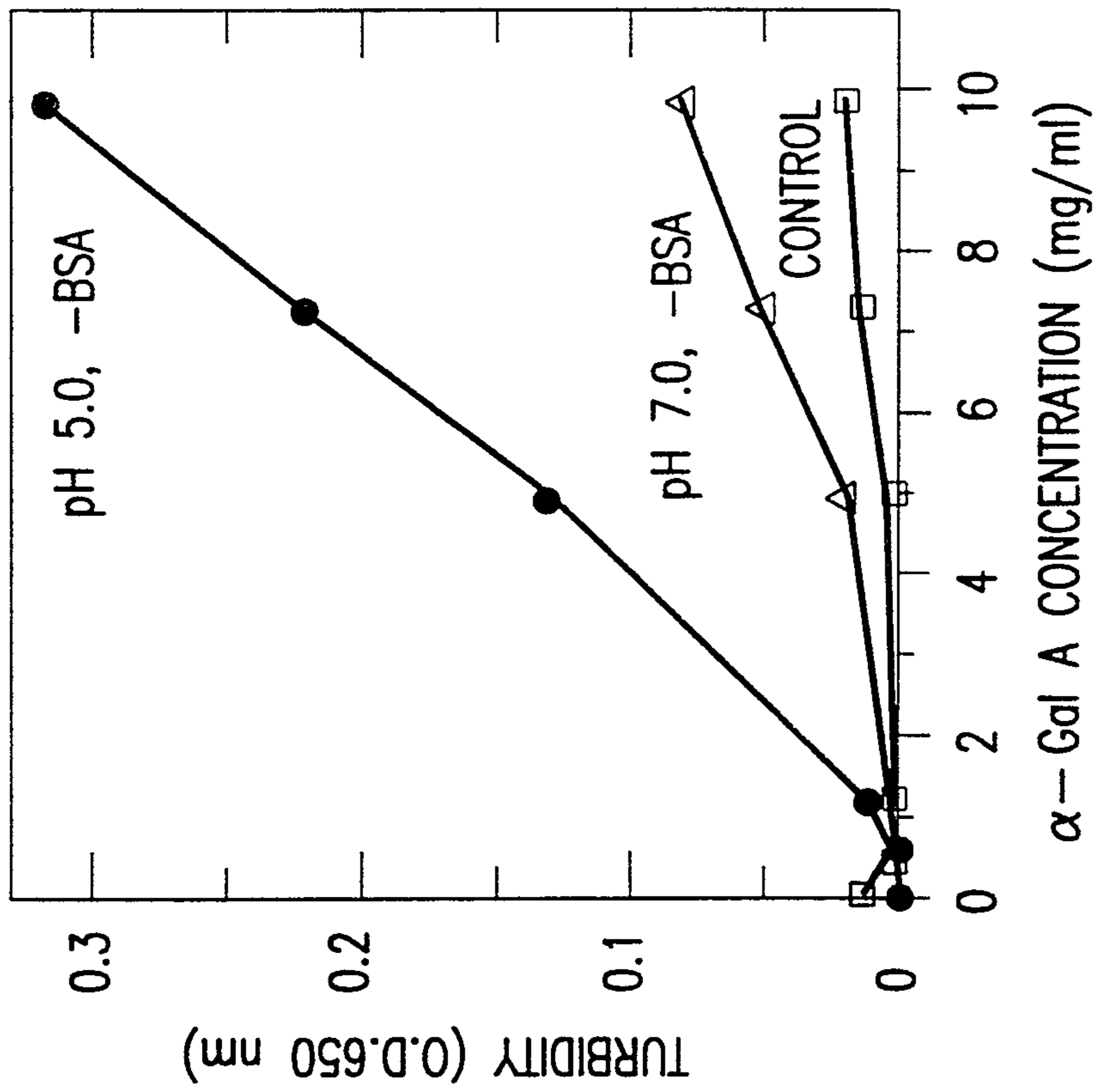


FIG.27B

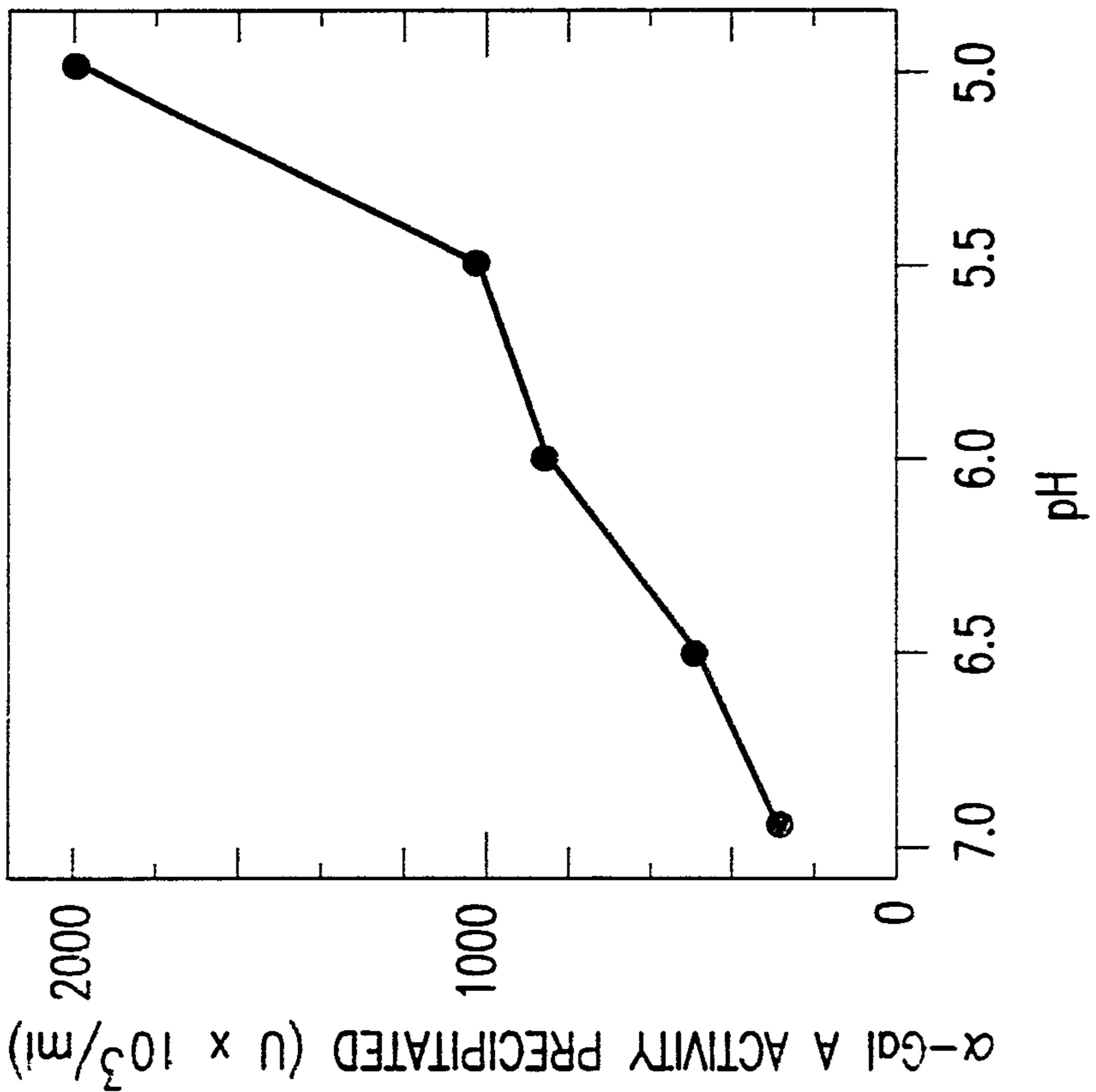


FIG.27A

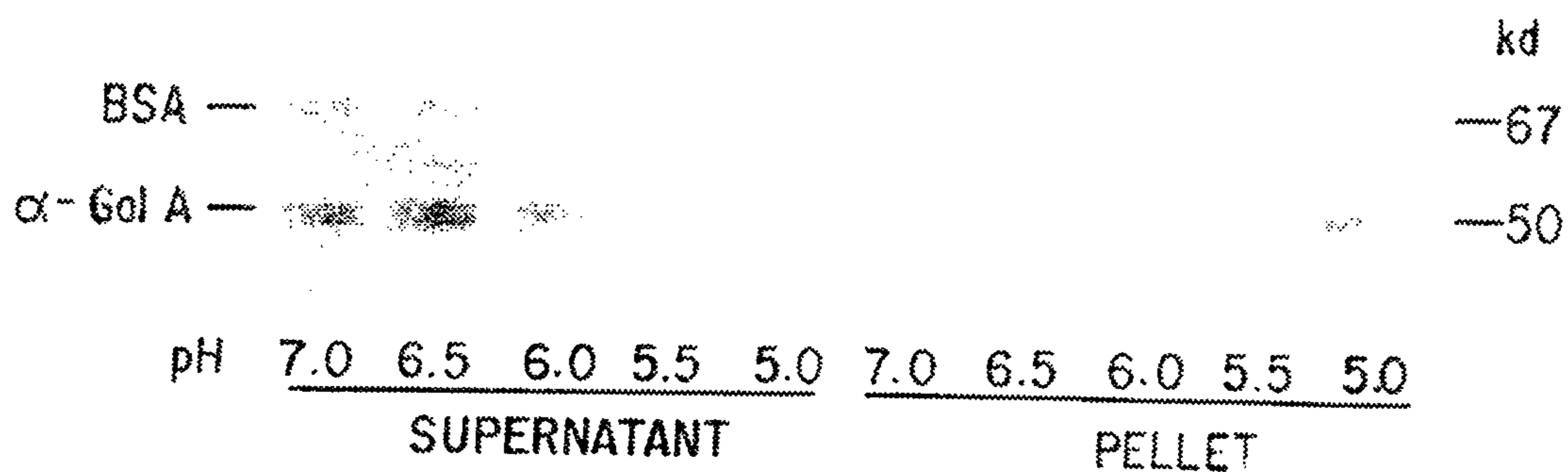


FIG. 27C

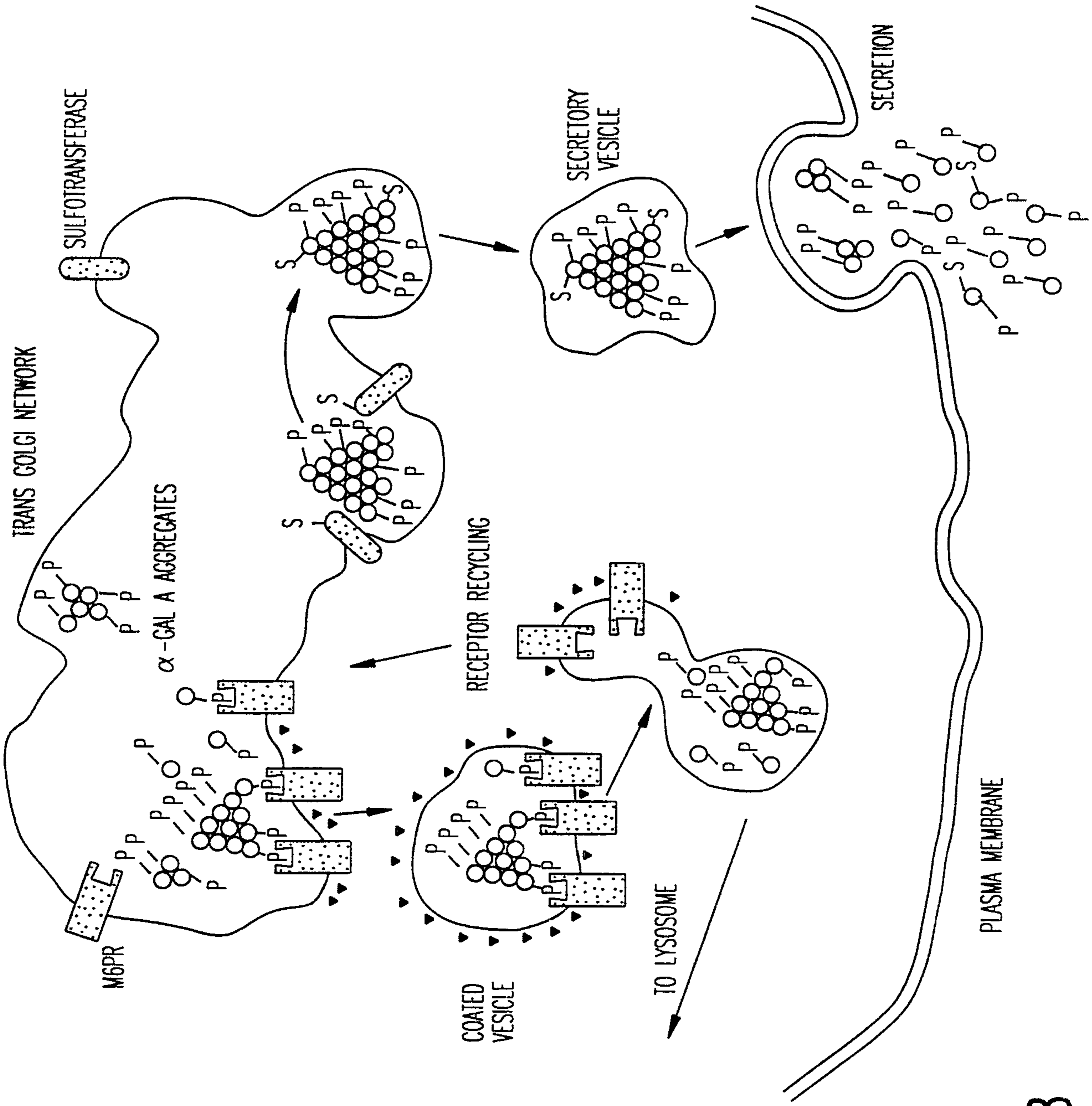


FIG.28