COMMONWEALTH OF A[illegible]

601190

Melb[illegible]

ONE HUNDRED DOLLARS SIXTY DOLLARS

Patents Act 1952-1969

CONVENTION APPLICATION FOR A PATENT

FEE STAMP TO VALUE OF $...160... ATTACHED
MAIL OFFICER...[illegible]

(1) Here insert (in full) Name or Names of Applicant or Applicants, followed by Address (es).

I/We[1] BEHRINGWERKE AKTIENGESELLSCHAFT

of D-3550 Marburg, Federal Republic of Germany

26.6.90

(2) Here insert Title of Invention.

hereby apply for the grant of a Patent for an invention entitled:[2]

A PROCESS FOR OBTAINING ACTIVE PROTEINS FROMA BIOLOGICALLY INACTIVE FORM

(3) Here insert number(s) of basic application(s)

which is described in the accompanying complete specification. This application is a Convention application and is based on the application numbered[3]

P36 18 817.4

(4) Here insert Name of basic Country or Countries, and basic date or dates

for a patent or similar protection made in[4] Federal Republic of Germany on 4th June 1986

My/Our address for service is Messrs. Edwd. Waters & Sons, Patent Attorneys, 50 Queen Street, Melbourne, Victoria, Australia.

DATED this 2nd day of June 19 87

(5) Signature (s) of Applicant (s) or Seal of Company and Signatures of its Officers as prescribed by its Articles of Association.

[5] BEHRINGWERKE AKTIENGESELLSCHAFT

by James Murray

James Murray

Registered Patent Attorney

LODGED AT SUB-OFFICE
- 3 JUN 1987
Melbourne

To: THE COMMISSIONER OF PATENTS.

Edwd. Waters & Sons, Melbourne.

Form 7

COMMONWEALTH OF AUSTRALIA

Patents Act 1952

DECLARATION IN SUPPORT OF A CONVENTION APPLICATION UNDER PART XVI. FOR A PATENT.

In support of the Convention application made under Part XVI. of the Patents Act 1952 by BEHRINGWERKE AKTIENGESELLSCHAFT of D-3550 Marburg, Federal Republic of Germany for a patent for an invention entitled:

A PROCESS FOR OBTAINING ACTIVE PROTEINS FROM A BIOLOGICALLY INACTIVE FORM

We, Heribert Bug of 7 Amselweg, D-3551 Niederweimar,
Philipp Stein of 28 Höhenweg, D-3550 Marburg,
Federal Republic of Germany

do solemnly and sincerely declare as follows:

1. We are authorized by BEHRINGWERKE AKTIENGESELLSCHAFT the applicant for the patent to make this declaration on its behalf.

2. The basic application(s) as defined by Section 141 of the Act was (were) made at München in the Federal Republic of Germany under No. P 36 18 817.4

on June 4, 1986

by BEHRINGWERKE AKTIENGESELLSCHAFT

3. Reinhard Hermann
30 Zilverberg
NL-2716 LZ-Zoetermeer
Netherlands

is/~~are~~ the actual inventor(~~s~~) of the invention and the facts upon which BEHRINGWERKE AKTIENGESELLSCHAFT is entitled to make the application are as follows:

The said BEHRINGWERKE AKTIENGESELLSCHAFT is the assignee of the said Reinhard Hermann

4. The basic application referred to in paragraph 2 of this Declaration was (were) the first application(s) made in a Convention country in respect of the invention the subject of the application.

DECLARED at Marburg, Federal Republic of Germany

this 22nd day of April 1987.

To the Commissioner of Patents

BEHRINGWERKE AKTIENGESELLSCHAFT

Prokurist
ppa. Bug

Prokurist
ppa. Stein

(12) PATENT ABRIDGMENT (11) Document No. AU-B-73796/87
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 601190

(54) Title
OBTAINING ACTIVE PROTEINS FROM BIOLOGICALLY INACTIVE FORMS

International Patent Classification(s)
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(71) Applicant(s)
BEHRINGWERKE AKTIENGESELLSCHAFT

(72) Inventor(s)
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(56) Prior Art Documents
EP 150066

(57)

The invention relates to a process for converting a protein from a conformation in which it is biologically inactive into a biologically active form. In the case of a denatured natural protein it would also be possible to call this process renaturation.

CLAIM

1. A process for the preparation of a spatial form, which has biological activity, of a protein from a biologically inactive spatial form, which comprises the protein being dissolved with the addition of a denaturing agent and thus converted into the random coil form, and the solution being made to pass through a material which has molecular sieve properties by a force exceeding the force of gravity and contains a reactivation buffer in which the protein will assume a spatial form which has biological activity, and this material having molecular sieve properties being selected so that the molecules of the denaturing agent can penetrate, but the protein molecules cannot.

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PATENTS ACT 1952-69

COMPLETE SPECIFICATION

(ORIGINAL)

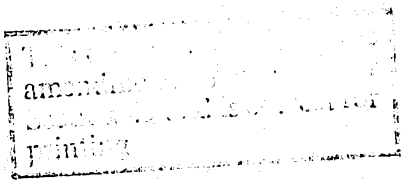
Class Int. Class

Application Number:
Lodged:

Complete Specification Lodged:
Accepted:
Published:

Priority :

Related Art :



Name of Applicant : BEHRINGWERKE AKTIENGESELLSCHAFT

Address of Applicant : D-3550 Marburg, Federal Republic of Germany

Actual Inventor: REINHARD HERMANN

Address for Service : EDWD. WATERS & SONS,
50 QUEEN STREET, MELBOURNE, AUSTRALIA, 3000.

Complete Specification for the invention entitled:

A PROCESS FOR OBTAINING ACTIVE PROTEINS FROM A BIOLOGICALLY INACTIVE FORM

The following statement is a full description of this invention, including the best method of performing it known to :- us

BEHRINGWERKE AKTIENGESELLSCHAFT

86/B 011 - Ma 559
Dr. Ha/Bn

A process for obtaining active proteins from a biologically inactive form

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The invention relates to a process for converting a protein from a conformation in which it is biologically inactive into a biologically active form. In the case of a denatured natural protein it would also be possible to call this process renaturation.

Background of the invention and prior art

Purification and sterilization processes applied to protein preparations may result in partial denaturation of the protein employed. Hitherto it was preferable, particularly based on economic considerations, to separate out and discard denatured protein. Protein prepared by gene manipulation in prokaryotes is largely in a biologically inactive form.

In order to raise the yield of "natural" protein, that is to say that with the correct spatial structure and the biological activity of the natural protein, it is necessary first for the polypeptide chain to be unfolded to give a random coil, and any incorrect disulfide bridges which are present to be reduced. This is normally carried out by incubation in at least 4 mol/l guanidine hydrochloride solution or at least 6 mol/l urea solution, where appropriate with the addition of a reducing agent such as dithiothreitol (DTT). Subsequently, the formation of the correct protein structure has, to date, been brought about by dilution (at least 1:40) or dialysis against a "physiological" buffer solution.

It is hardly possible to use either method industrially.

Dilution of volumes which are large at the outset, followed by reconcentration, is time-consuming, troublesome and costly. This is similarly true of dialysis of large volumes. Furthermore, slow removal of denaturing agent considerably reduces the reactivation yield because side-reactions, such as aggregations, take place preferentially in the intermediate range of concentrations of denaturing agent.

Detailed description of the invention

It has been found, surprisingly, that the disadvantages of the processes of the prior art can be avoided by removing the denaturing agent from the solution containing the denaturing agent and the protein by allowing the solution to pass through a material which has molecular sieve properties and which contains a medium in which the protein assumes its biologically active form, the selected pore size of this molecular sieve material being such that the denaturing agent can penetrate, but the protein cannot.

Thus the invention relates to a process for the preparation of a spatial form, which has biological activity, of a protein from a spatial form which is biologically inactive, which comprises the protein being dissolved with the addition of a denaturing agent and thus converted into the random coil form, and the solution being allowed to pass through a material which has molecular sieve properties and contains a liquid medium in which the protein can assume its spatial form which has biological activity, and this material having molecular sieve properties being selected so that the molecules of the denaturing agent can penetrate, but the protein molecules cannot.

Examples of possible locations of the molecular sieve are a column or a centrifuge basket.

Once the molecular sieve has been equilibrated with the medium in which the protein can assume a biologically active form it is preferable for the portion of the medium which is not located in the pores of the molecular sieve (the "external volume") to be removed. This is expediently achieved by centrifugation, but can also be brought about by, for example, blowing out with a gas or sucking out by applying a vacuum.

The solution which contains the "unfolded" protein and the denaturing agent is then applied to the molecular sieve. The penetration of the solution through the molecular sieve material should be effected by a force exceeding the force of gravity. Centrifugation is preferred for this, but it is also effected by gas pressure or vacuum. When centrifugation is employed the operating procedure substantially corresponds to the known techniques of basket or screen centrifugation.

A molecular sieve of this type may be one of the materials which are known for gel filtration and which is chemically resistant to the denaturing agent, for example ^{R}Sephadex G-25, DG 6P (^{R}Bio Rad, USA) or controlled pore glass. The pore size is selected so that the denaturing agent can penetrate into the matrix, but the protein cannot. The exclusion limit will usually be at a M_r of 6,000 to 10,000 (M_r = molecular weight).

It is equilibrated with a solution in which the protein assumes its active form, preferably with a buffer, and transferred, for example, into a column, which can preferably be centrifuged, or into a centrifuge basket. The solution not located in the pores of the matrix (not "in the internal volume") is preferably removed by centrifugation at about 300-1,000xg. The protein solution containing the denaturing agent is then applied (volume less than 30% of the gel volume). Whereas molecules of the denaturing solution can replace the buffer in the internal

volume, proteins (molecular weight above 6,000) remain in the external volume. It is possible by renewed centrifugation (2 min, 300-1,000xg) to spin the proteins quantitatively into a collecting vessel. This can be carried out by centrifugation in a basket centrifuge in accordance with known desalination processes. No denaturing agents are detectable thereafter. The volume of the resulting solution then corresponds to the volume of the solution applied.

The removal of the equilibration medium in the external volume, as well as the speeding up of the replacement of the equilibration medium in the internal volume by the denaturing agent contained in the protein solution, can also be brought about by gas pressure or vacuum.

The process according to the invention makes it possible to transfer, rapidly, quantitatively and without dilution, a protein even from large volumes of a denaturing medium into a medium in which the protein assumes an active form, and to obtain high yields of active protein.

It is possible in the manner described for protein material which cannot otherwise be exploited to be rendered commercially utilizable.

The process is distinguished by simplicity, rapidity and reproducibility. It is possible to carry it out using available and conventional equipment and materials. After use, the gel material can be regenerated and, for example, guanidine hydrochloride can be recovered. The protein concentration remains unchanged.

Denatured proteins are, specifically, proteins in an unnatural state after a heat treatment, for example for inactivation of infectious material, after acid treatment, for example acid cleavage of fusion proteins obtained by gene manipulation, after treatment with structure-damaging

agents, for example during the course of purification, extraction or solubilization steps and on inactivation of infectious material, or after preparation by gene manipulation resulting in an incorrect conformation and/or incorrect formation of disulfide bridges.

Examples of suitable denaturing agents for complete unfolding of the protein are high-molarity solutions of guanidinium salts, urea or other chaotropic molecules, where appropriate in the presence of a reducing agent, for example 50-150 mmol/l dithiothreitol (DTT). Examples of the usual concentrations are for guanidine salts 4-7, for urea 6-8 and for isothiocyanate 6-8 mol/l and for 2-chloroethanol about 400 ml/l.

The development of the biologically active (natural) structure is brought about by rapid transfer into a buffer which favors the natural structure.

The rapidity of the transfer is important for a high yield. In the process described, the time is in the range of seconds to minutes.

Examples of suitable activating buffers are phosphate or tris buffer or buffers known as "Good buffers" (Biochem. (1966) 15, 467-477) which are adjusted to the pH of maximum activity or stability of the protein.

The denaturing molecules are rapidly and quantitatively removed, preferably by centrifugation.

The medium in which the protein assumes its biologically active conformation is usually a buffer and has a composition which is advantageous for the stability of the protein (contains, for example, phosphates, sulfates, citrates). Examples of other additives it can contain are sugars, peptides or proteins to stabilize the natural structure, or detergents, for example ^{R}Tween 20 or NP40

to prevent adhesion or aggregation and/or for solvation, and/or SH reagents or redox systems, for example DTT or glutathione/glutathione disulfide (GSH/GSSG) to set up the redox potential which is optimal for the formation of correct disulfide bridges.

Reproducible redox conditions are ensured by degassing the buffers and saturating with nitrogen.

Basket centrifugation within the meaning of the invention is every centrifugation technique in every volume range with any equipment, in which any desired macromolecule (in buffer A) is transferred by centrifugation through a gel filtration medium, which has been equilibrated with buffer B and optionally precentrifuged, into buffer B.

The examples which follow illustrate the invention.

Example 1

Denaturation of active mouse GM colony stimulating factor (Mu GM-CSF, recombinant from yeast) in guanidine, and reactivation.

3 samples, each comprising 1 µg, of each of 5 solutions of GM-CSF of 5 different degrees of glycosylation (A to E) were taken up in 40 µl of 6 mol/l guanidine.HCl in phosphate-buffered saline (PBS), pH 7.2, and the solution was kept at room temperature for 60 minutes.

^{R}Sephadex G-25 was packed into 15 tubes with a volume of 0.5 ml, and groups of 5 were equilibrated with degassed, nitrogen-saturated PBS containing no additive or containing 1 mmol/l DTT or 0.02 ml/100 ml ^{R}Tween 20. The liquid in the external volume was spun out at 700xg (5 minutes). In each case, one of the 5 solutions of GM-CSF (A to E) was applied to one of these 5 tubes equilibrated with PBS or with PBS + DTT or Tween.

Immediately after the 15 different solutions had been applied to the 15 tubes they were centrifuged at 700xg for two minutes and 15 samples of 40 µl of a guanidine-free GM-CSF preparation were obtained.

All 15 samples were stored overnight at room temperature under nitrogen, and then the activity was determined in the bone marrow test or on a GM-CSF-dependent cell line.

The activity of the guanidine-treated samples depended on the reactivation buffer and ranged up to 100% of the initial activity (about 2×10^7 units (U)/mg). The yield of protein, determined by SDS electrophoresis and Western blot, was virtually quantitative.

The results are shown in Figure 1. In this diagram, in each case 1 designates the column for the activity of the solution of GM-CSF which has not been treated with denaturing agent and reactivated, and 2 designates that for the GM-CSF reactivated in PBS, 3 that in PBS and Tween®, and 4 that in PBS and DTT. A to E each designate one group of activities for one of five GM-CSF preparations with differing extents of glycosylation.

Example 2

Unfolding in 6 mol/l guanidine and activation of aggregated recombinant human GM-CSF.

2 preparations of aggregated, freeze-dried human GM-CSF (A and B), which had been obtained by acid cleavage of a fusion protein from E. coli, were each dissolved in 6 mol/l guanidine.HCl in PBS and incubated at room temperature for 60 minutes. The CSF contribution to the total protein was about 20 micrograms/100 micrograms.

Subsequent treatment was carried out as in Example 1.

The results are shown in Fig. 2.

The activity of the guanidine-treated samples reached an activity which was up to 130 times the initial activity. The maximum specific activity was determined to be 2×10^7 units/mg. The reactivation buffers used were PBS (columns No. 1), PBS + 0.02% Tween® 20 (columns No. 2) or PBS + 0.1 mmol/l dithiothreitol (columns No. 3).

Example 3

Unfolding in 8 mol/l urea and activation of aggregated, inactive recombinant human GM-CSF

Aggregated, freeze-dried human GM-CSF from E. coli (3 samples after acid cleavage designated A, B and C, and one sample which was not cleaved and was designated D; CSF content about 20 µg/100 µg of total protein) was dissolved in 8 mol/l urea in tris.HCl, pH 8.0 (protein concentration 1 mg/ml, volume of each sample 0.5 ml) and incubated at room temperature for 60 min. The subsequent treatment was carried out as in Example 1. The reactivation buffer used was PBS (columns No. 1) or PBS + 0.02% Tween (columns No. 2), PBS + "low"* GSH (columns No. 4) or PBS + "high"* GSH (columns No. 3) after acid cleavage. In all cases specific activity near to or the same as the maximum specific activity was obtained from completely inactive material (Fig. 3).

Fusion protein before cleavage also shows considerable biological activity (D). The specific activity after reactivation was determined to be 1-2 x 10^7 units/mg.

* "low" GSH: 25 µM GSH/50 µM GSSG (corresponds to the extracellular redox potential)
"high" GSH: 5 mM GSH/0.1 mM GSSG (corresponds to the intracellular redox potential)

Example 4

Unfolding and complete reduction of all the disulfide bridges in aggregated, inactive, recombinant human GM-CSF, refolding and reoxidation to give the biologically active material as in Example 3.

Denaturation and reduction in 8 mol/l urea in tris.HCl of pH 8.0 + 0.15 mol/l dithiothreitol; folding to give the biologically active product in PBS (columns No. 1) or PBS + 0.1% human serum albumin (columns No. 2), PBS + high GSH (columns No. 3) or PBS + low GSH (columns No. 4) (Fig. 4).

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A process for the preparation of a spatial form, which has biological activity, of a protein from a biologically inactive spatial form, which comprises the protein being dissolved with the addition of a denaturing agent and thus converted into the random coil form, and the solution being made to pass through a material which has molecular sieve properties by a force exceeding the force of gravity and contains a reactivation buffer in which the protein will assume a spatial form which has biological activity, and this material having molecular sieve properties being selected so that the molecules of the denaturing agent can penetrate, but the protein molecules cannot.

2. The process as claimed in claim 1, wherein the portion of the reactivation buffer which is not located in the internal volume of the material having molecular sieve properties and in which the protein will assume a biologically active spatial form is removed by centrifugation, blowing or sucking out before the protein solution is contated with this material.

3. The process as claimed in claim 1, wherein the rate of passage of the protein containing solution is increased by centrifugation.

4. The process as claimed in claim 1, wherein the material having molecular sieve properties is a material which is known for gel filtration and is chemically resistant to the denaturing agent.

5. The process as claimed in claim 1, wherein the material having molecular sieve properties is ^{R}Sephadex G-25, ^{R}BioRad DG 6P or controlled pore glass.

6. The process as claimed in claim 1, wherein the reactivation buffer in which the protein assumes a biologically

active form is an aqueous buffer which contains agents favorable to the activity and stability of the protein, SH reagents or wetting agents.

DATED this 2nd day of June 1987.

BEHRINGWERKE AKTIENGESELLSCHAFT

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FIG.1

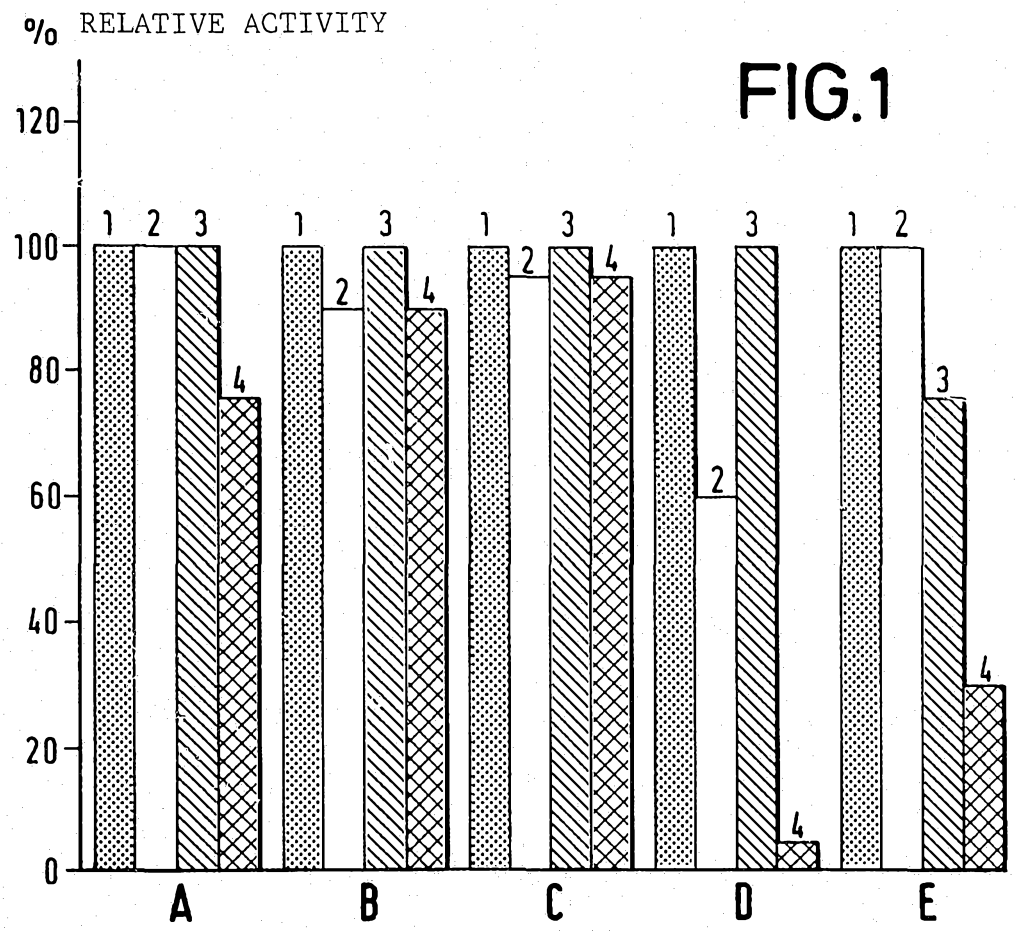
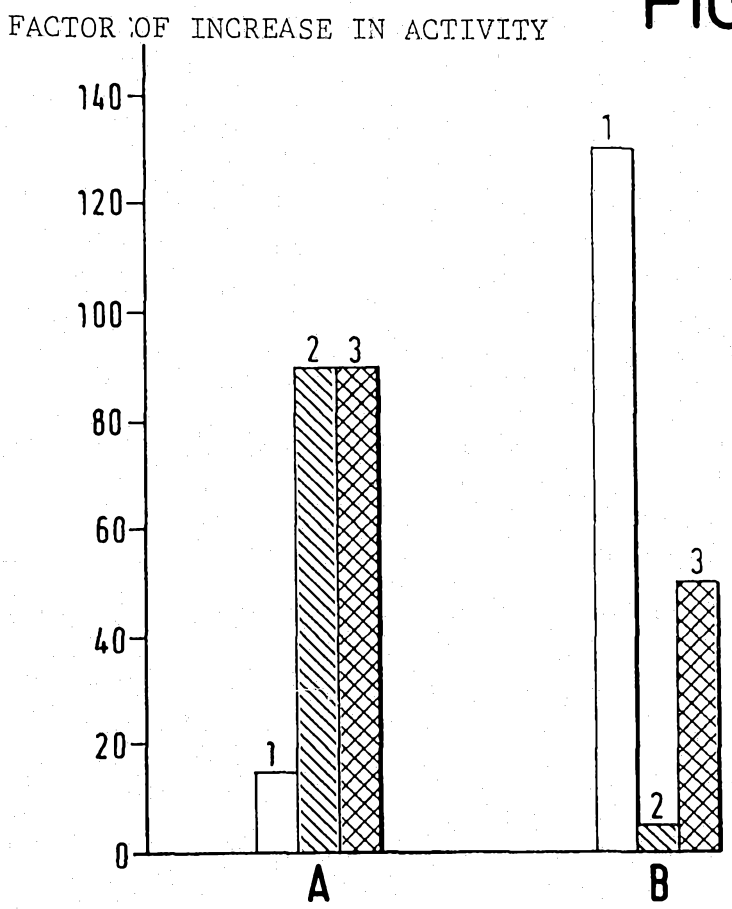


FIG. 2



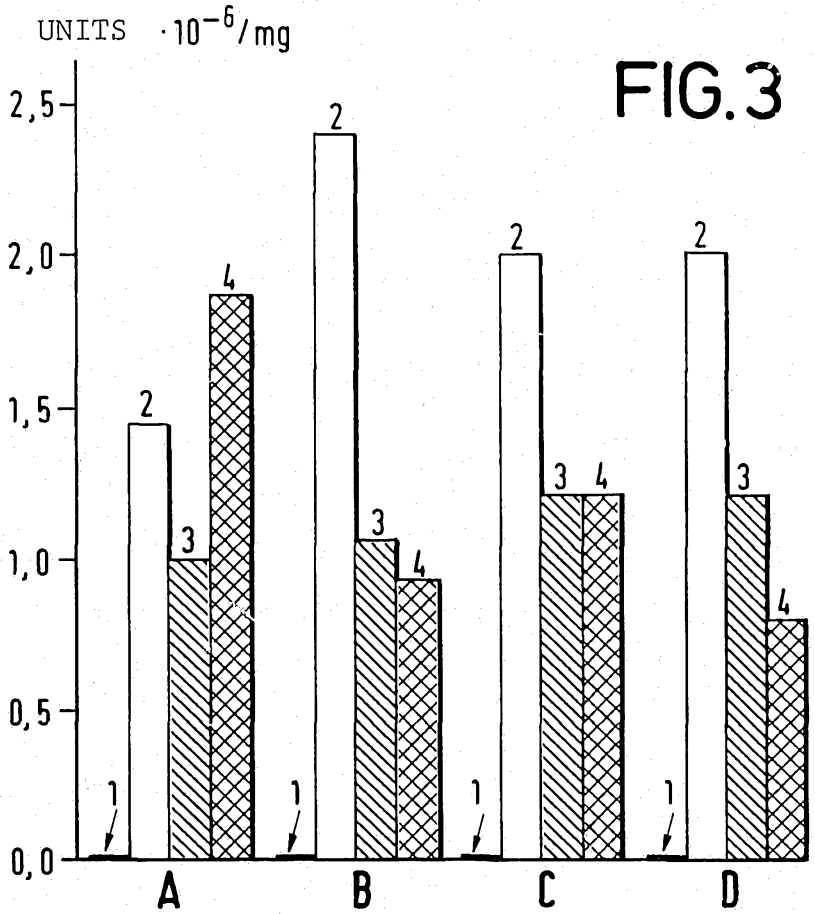


FIG.3

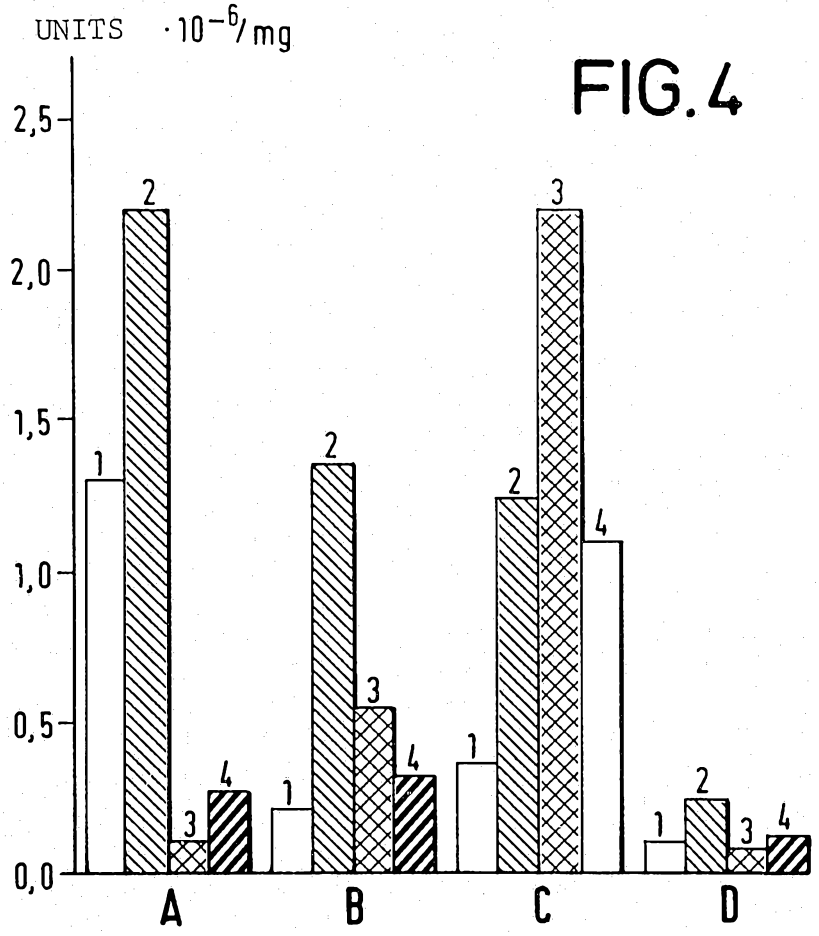


FIG. 4