

(12) PATENT
(19) AUSTRALIAN PATENT OFFICE

(11) Application No. AU 199894060 B2
(10) Patent No. 714264

(54) Title
Improved process to stabilize proteins

(51)⁶ International Patent Classification(s)
A61K 047/02 A61K 039/395
A61K 009/08

(21) Application No: 199894060 (22) Application Date: 1998 .11 .20

(30) Priority Data

(31) Number	(32) Date	(33) Country
EP97120528	1997 .11 .22	CH
EP98102846	1998 .02 .19	CH

(43) Publication Date : 1999 .06 .10
(43) Publication Journal Date : 1999 .06 .10
(44) Accepted Journal Date : 1999 .12 .23

(71) Applicant(s)
Boehringer Mannheim GmbH

(72) Inventor(s)
Klaus Hellerbrand; Apollon Papadimitriou; Gerhard Winter

(74) Agent/Attorney
SPRUSON and FERGUSON,GPO Box 3898,SYDNEY NSW 2001

(56) Related Art
AU 24094/92
AU 16339/97
EP 314095

Improved Process to Stabilize Proteins

ABSTRACT

An improved process for preventing the formation of protein aggregates in a reconstituted lyophilisate of a pharmaceutical composition of a protein, in which an aqueous buffered solution of the protein is frozen, thawed, divided into compartments of injectable amounts and these compartments are lyophilized which is characterized in that the aqueous buffered solution of the protein contains potassium phosphate buffer as a buffer substance and the ratio of potassium to sodium ions in the solution is at least 10:1, can advantageously be used to produce stable lyophilisates of pharmaceutical compositions.



AUSTRALIA
PATENTS ACT 1990

COMPLETE SPECIFICATION

FOR A STANDARD PATENT

ORIGINAL

Name and Address
of Applicant: Boehringer Mannheim GmbH
116 Sandhofer Strasse
D-68305 Mannheim
GERMANY

Actual Inventor(s): Klaus Hellerbrand, Apollon Papadimitriou and Gerhard
Winter

Address for Service: Spruson & Ferguson, Patent Attorneys
Level 33 St Martins Tower, 31 Market Street
Sydney, New South Wales, 2000, Australia

Invention Title: Improved Process to Stabilize Proteins

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

The invention concerns an improved process for stabilizing proteins in a freezing or lyophilization process or during storage at low temperatures.

Proteins such as enzymes or antibodies as well as fragments
5 thereof are unstable and susceptible to loss of activity and/or to formation of soluble or insoluble aggregates in aqueous solutions and when stored at low temperatures (below 0°C) and in particular in repeated freezing and thawing processes and these aggregates become apparent by forming
10 particles and thus as turbidities. However, such aggregate and/or particle formation cannot be tolerated or at least only in traces for pharmaceutical compositions of proteins. A pharmaceutical composition should be a clear solution and if it is present as a lyophilisate it should also lead to a
15 clear particle-free solution when reconstituted which is also free of soluble protein aggregates.

Numerous processes and additives are known for the stabilization of proteins in solutions. For example the stabilization of proteins by adding heat-shock proteins such
20 as HSP25 is for example described in EP-A 0 599 344. The stabilization of antibodies by adding block polymers composed of polyox propylene and polyoxy-ethylene and by phospholipids is described in EP-A 0 318 081. EP-A 0 025 275 describes the stabilization of immunoglobulins by adding a salt of a basic
25 substance containing nitrogen such as arginine, guanidine or imidazole. Other suitable additives for stabilization are polyethers (EP-A 0 018 609), glycerin, albumin and dextran sulfate (US Patent 4,808,705), detergents such as Tween®²⁰ (DE 26 52 636, GB 8514349), chaperones such as GroEL
30 (Mendoza, J.A. Biotechnol. Tech. 10 (1991) 535 - 540), citrate buffer (WO 93/22335) or chelating agents (WO 91/15509). Although these additives enable proteins to be stabilized to a certain extent in aqueous solutions. It has, however, turned out that none of the processes known in the

prior art is suitable for stabilizing proteins during repeated freezing and thawing processes in such a way that no soluble or insoluble aggregates or only negligible amounts for therapeutic purposes are formed during rethawing, during storage at temperatures below 0°C or when a solution is reconstituted after lyophilization.

In EP-A 0 314 095 a lyophilisate of a plasma protein such as factor VIII is described which contains histidine buffer as a buffer substance and calcium chloride as an additive and is present in a high ionic strength (0.35 to 1.2 mmol/l NaCl).

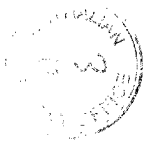
A lyophilisate of a plasma protein such as factor VIII is described in EP-A 0 315 968 which contains 0.5 to 15 mmol/l sodium chloride or potassium chloride, 0.01 to 10 mmol/l lysine hydrochloride and 0.2 to 5 mmol/l histidine as a buffer ion. However, histidine buffer is not suitable for stabilizing proteins and for preventing aggregate and particle formation when lyophilisates of proteins are reconstituted.

Consequently the object of the invention is the provision of a process which can substantially prevent aggregate and particle formation when lyophilisates of pharmaceutical compositions of proteins are reconstituted.

Hence a first aspect of the present invention concerns an improved process for preventing the formation of protein aggregates of more than 6000 particles in the range of above 10µm and 600 particles in the range of 25µm per injection dose in a reconstituted lyophilisate of a pharmaceutical composition of a protein, in which process an aqueous buffered solution of the protein is frozen, thawed, divided into compartments of injectable amounts and these compartments are lyophilized, wherein the aqueous buffered solution of the protein contains potassium phosphate buffer as a buffer substance and as a substance to avoid protein aggregates and the ratio of potassium to sodium ions in the solution is 10:1 or higher. The aqueous buffer solution preferably contains essentially no sodium ions.

A second aspect of the present invention provides a meltable solid storage form of an injection dose of a protein containing as protein aggregates a maximum of 6000 particles in the range above 10µm and 600 particles in the range of 25µm per injection dose, wherein it is essentially amorphous, contains a frozen solution of the protein with potassium phosphate buffer as the only buffer substance and as the only substance to avoid protein aggregates and the ratio of potassium ions to sodium ions is at least 10:1.

A third aspect of the present invention provides a pharmaceutical composition of a protein in an aqueous buffered solution in the pH range between 6 and 8, wherein the solution contains potassium phosphate buffer as the only buffer substance and as the only substance to avoid formation of protein aggregates of more than 6000 particles in the range



2a

of 10µm and 600 particles in the range of 25µm per injection dose in the reconstituted lyophilisate of said pharmaceutical composition and

- a) the ratio of potassium to sodium ions in the solution is 10:1 or higher,
- b) the buffer concentration is between 10 and 300 mmol/l.

0
0
0
0
0

0
0
0
0
0



The invention enables pharmaceutical compositions of proteins, in particular proteins which have a tendency to dimerize or multimerize such as antibodies, to be formulated into a stable pharmaceutical composition in a neutral pH range (pH 6 - 8, preferably pH 6.5 - 7.5). Proteins such as antibodies tend to aggregate in the neutral pH range especially if the solutions are frozen (optionally lyophilized) once or several times and thawed again.

10 A pharmaceutical composition is especially advantageous in potassium phosphate buffer in the pH range between 6 and 8, at a buffer concentration between 10 and 300 mmol/l, preferably between 50 and 250 mmol/l in which the lowest possible number of sodium ions are present in the pharmaceutical composition. A suitable ratio of potassium to 15 sodium ions in the solution is 10:1 or more. It is particularly preferable that potassium phosphate buffer is used alone as the buffer substance in the pharmaceutical composition and no sodium salt (such as e.g. sodium chloride) is added. In such a case almost no sodium ions are present in 20 the pharmaceutical composition or it only contains them in such low amounts that they do not cause formation of aggregates of proteins during repeated freezing or thawing.

It has turned out that lyophilisates of protein solutions which have been frozen at least once during the production process can then be reconstituted substantially without formation of turbidities if potassium phosphate buffer is used as the buffer substance. The usual buffers such as sodium phosphate buffer, histidine buffer or citrate buffer lead to the formation of aggregates in such a process which 30 are mainly composed of the protein and thus also lead to turbidities to a considerable degree. The frozen protein solutions are already completely frozen through below ca. -15°C, have eutectic points above ca. -15°C and can thus already be stored at this temperature or at lower 35 temperatures preferably e.g. at -20°C. Since a solution is only completely frozen through below the eutectic temperature, this means that a protein in a phosphate buffer

containing sodium ions is subjected to a higher stress during the frozen storage (usually at -20°C) and during the freezing/thawing process than in a buffer free of sodium ions or in a buffer in which the sodium ion concentration is very low. According to the invention this stress is avoided in the above-mentioned formulations resulting in a suppression of aggregate and particle formation. This formulation enables a stable storage of the protein solution at -20°C which can save costs. Potassium phosphate buffers in contrast to sodium phosphate buffers have only a slight pH shift (preferably at most ± 1 pH unit, particularly preferably at most ± 0.5 pH units) during the freezing process.

It has turned out that the concentration of the phosphate buffer should be at least 10 mmol/l, preferably about 50 mmol/l or higher in order to effectively prevent particle formation. Since the osmolarity should not be too high (it should advantageously be in the physiological range, preferably ca. 300 mOsm after reconstitution (± 20 mOsm, a range of 100 to 500 mOsm is also suitable)) in pharmaceutical compositions (i.e. preferably in the reconstituted solution), the concentration of the buffer substance or optionally the sum of buffer substance and salt should be not more than 250 - 300 mmol/l. The buffer concentration is preferably between 50 and 250 mmol/l in the compartment. However, higher concentrations of buffer substance and/or salt can be tolerated in the production of the solutions (bulkware) used to produce the compartments.

If a salt additive is desired in the pharmaceutical composition especially to adjust the ionic strength, it is advantageous according to the invention to also not use sodium salts or to select a concentration of the sodium ions which is substantially lower than the concentration of the potassium ions. It is therefore expedient to add a potassium salt such as potassium chloride instead of the otherwise usual sodium chloride. However, it has turned out that low amounts of sodium salts (e.g. ca. 10 mmol/l or less) do not interfere provided the ratio of potassium ions to sodium ions is 10:1

or higher. It is not possible to add calcium salts such as e.g. calcium chloride since calcium phosphate is precipitated by such an addition and hence, apart from the formation of undesired turbidity, the buffer effect of the potassium phosphate according to the invention is abolished.

Non-soluble aggregates whose formation should be prevented in the process according to the invention are essentially understood as protein aggregates whose size is usually at least 1 μm but can also be in the range above 10 μm . The particles can be determined by suitable particle counting methods using commercial particle counting instruments such as e.g. the particle counting instrument AccuSizer 700 from PSS (Particle Sizing Systems, USA). According to the invention an improvement of the process is achieved when the number of particles between 2 and 400 $\mu\text{m}/\text{ml}$ is < 3000 or the number of particles between 10 and 400 $\mu\text{m}/\text{ml}$ is 2000 or less. According to the USP (US-Pharmacopoeia) a maximum of 6000 particles in the range above 10 μm and a maximum of 600 particles in the range above 25 μm are allowed per injected dose of a pharmaceutical preparation. This can be achieved according to the invention in a simple manner for therapeutic compositions of proteins.

Proteins (polypeptides) are understood within the sense of the invention as proteins or protein fragments as well as chemically modified proteins. Proteins which are desirably stabilized for pharmaceutical compositions are preferably antibodies, antibody fusion proteins such as immunotoxins, enzymes and protein hormones such as erythropoietin, somatostatin, insulin, cytokines, interferons or plasminogen activators.

Compartments within the sense of the invention are understood as aliquots of the protein solution which, optionally after further processing (addition of further pharmaceutically acceptable substances), are suitable as pharmaceutical compositions preferably for injection in the patients.

The pH range in which the pharmaceutical composition is stabilized by the potassium phosphate buffer is preferably a slightly acidic, neutral or slightly alkaline range (ca. pH 6-8, preferably about pH 7).

- 5 According to the invention it is preferable to add a nonionic detergent such as polysorbate (e.g. Tween[®] 80), preferably at a concentration of at most 0.1 % by weight and at least 0.01 % by weight.

- 10 In addition it is preferably to add cryoprotectors or glass formers such as a non-reducing sugar (preferably sucrose or trehalose), advantageously at a concentration of at least 10 mg/ml, preferably of ca. 30 - 100 mg/ml.

- 15 Consequently a further subject matter of the invention is a low aggregate, meltable solid storage form of a protein which is essentially amorphous and is composed of a frozen solution of the protein and potassium phosphate buffer as the main buffer substance in which the ratio of potassium ions to sodium ions in the solution is at least 10:1.

- 20 Independent of the concentration of potassium ions and the residual content of sodium ions, the ratio of potassium to sodium ions should be at least 10:1, preferably at least 50:1. It is particularly preferable to use essentially sodium-ion-free potassium buffer.

- 25 In a further preferred embodiment of the invention the pharmaceutical composition contains a protein which has been produced by an in vitro cell culture (for example recombinant production or culture of a hybridoma cell line to produce monoclonal antibodies). In this case it is expedient to either add potassium salt and/or potassium phosphate buffer
30 with the first addition of salt or/and buffer, or to rebuffer at a later time in the isolation and purification process. This enables the interim stable storage of the polypeptide preparation below 0°C. Rebuffering is understood as an

exchange of ions for example by dialysis. In the purification and isolation process of the protein the buffer or salt concentration can indeed be higher than 50 - 100 mmol/l before compartmentation since these compositions are not used therapeutically. However, it is essential that an osmolarity that is suitable for an injectable composition is adjusted before the compartmentation.

The following examples, publications and figures further elucidate the invention, the protective scope of which results from the patent claims. The described processes are to be understood as examples which still describe the subject matter of the invention even after modifications.



Fig. 1 shows the determination of the eutectic points of various buffers and salt solutions.

15 Fig. 2 shows the shift of the pH value during freezing of phosphate buffers



Fig. 3 shows the particle formation of solutions of an antibody (against L-selectin) in various buffer solutions (A, B, C) after shear or freeze/thaw stress.

20 A: AB in 10 mmol/l KP, 150 mmol/l NaCl, pH 7; B: AB in 100 mmol/l KP, pH 7.2; C: AB in 100 mmol/l KP 0.01 % by weight Tween®80, pH 7.2;
a: centrifuged (starting material); b: after shearing stress (30 sec vortexing); c: after six freeze/thaw cycles (-20°C).

25
30 Fig. 4 shows the particle formation of solutions of an antibody against HBV in various buffer solutions (A, B, C) after shear or freeze/thaw stress.
A: AB in 10 mmol/l KP, 30 mmol/l NaCl, pH 6.5;
B: AB in 100 mmol/l KP, pH 7.2; C: AB in 100 mmol/l KP, 0.01 % by weight Tween®80, pH 7.2;

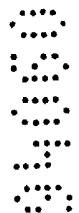
Fig. 5 shows the size exclusion HPLC analysis of soluble aggregates in protein solutions (humanized IgG according to example 3) after storage at temperatures below 0°C.

- 5 A: AB in 10 mmol/l KP, 150 mmol/l NaCl, pH 7.0;
 B: AB in 100 mmol/l KP, pH 7.2.

Example 1

Eutectic temperatures of various buffer and salt solutions

10 From Fig. 1 it is clear that the eutectic temperature of NaCl-containing buffers is ca. 10°C lower than that of NaCl-free buffers or solutions which contain KCl instead of NaCl. Since a solution is only completely frozen through below the eutectic temperature, this means that a protein in an NaCl-containing phosphate buffer is subjected to a higher stress than in NaCl-free buffer during frozen storage (usually at -20°C) and during the freeze/thaw process. According to the invention this stress is avoided in the above-mentioned formulations which suppresses the formation of aggregates and particles. This formulation enables a stable storage of the protein solution at -20°C by which means cost savings can be achieved.



Example 2

Shift of the pH value during freezing of phosphate buffers

25 It is clear from Fig. 2 that in NaCl-containing phosphate buffers the pH value greatly decreases during the freezing process due to precipitated disodium hydrogen phosphate. The pH value remains largely constant in NaCl-free potassium phosphate buffer.



Example 3

Particle formation in protein solutions after shear or freeze/thaw stress

Solutions of a humanized IgG (antibody against L-selectin) in various buffers (A, B, C) were analysed for particle content (Accu Sizer, Particle Sizing Systems, USA):

- A) AB in 10 mmol/l KP, 150 mmol/l NaCl, pH 7
 - B) AB in 100 mmol/l KP, pH 7.2
 - C) AB in 100 mmol/l KP, 0.01 % by weight Tween[®]80, pH 7.2
- 10 a) centrifuged (starting material)
b) after shear stress (30 sec. vortexing)
c) after six freeze/thaw cycles (-20°C)

The data in Fig. 3 each refer to 0.7 ml sample.

- It can be seen from Fig. 3 that particle formation is suppressed according to the invention by using sodium-free potassium phosphate buffers. This effect can be increased by the addition of a nonionic detergent (Tween[®]80, 0.01 % by weight).

Example 4

Particle formation in protein solutions after shear or freeze/thaw stress

Solutions of an antibody against HBV in various buffers (A, B, C) were analysed for particle content (Accu Sizer, Particle Sizing Systems):

- A) AB in 10 mmol/l KP, 30 mmol/l NaCl, pH 6.5
 - B) AB in 100 mmol/l KP, pH 7.2
 - C) AB in 100 mmol/l KP, 0.01 % by weight Tween[®]80, pH 7.2
- 25 a) centrifuged (starting material)
b) after shear stress (30 sec. vortexing)
30 c) after six freeze/thaw cycles (-20°C)

The data in Fig. 3 each refer to 0.7 ml sample.

It can be seen from Fig. 4 that particle formation is suppressed according to the invention by using sodium-free

potassium phosphate buffers. This effect can be increased by the addition of a nonionic detergent.

Example 5

Prevention of the formation of soluble aggregates during the storage of protein solutions (humanized IgG according to example 3) at temperatures below 0°C

Protein solutions were stored for several weeks at -20°C in A) 10 mM potassium phosphate, 150 mM NaCl, pH 7.0, and B) in 100 mM potassium phosphate, pH 7.2. Analysis of the soluble aggregates and the native protein was carried out by size exclusion HPLC (Fig. 5). According to the invention considerably fewer protein aggregates occur in the NaCl-free buffer than in the NaCl-containing buffer. This is above all due to the fact that a shift of the pH value is substantially prevented in the NaCl-free buffer and the storage temperature is considerably below the eutectic temperature. (see also examples 1 and 2).

Example 6:

Particle formation in protein solutions after freeze/thaw stress

The antibodies MAB L-selectin, MAB HBV; MAB PDGF-R and MAB LINGF-R in various buffers were analysed for particle content before and after freeze/thaw stress (6 x freezing/thawing) (Accu Sizer, Particle Sizing Systems) (results cf. table 1, C_{prot}: protein concentration). Particles with a size of 2-400 µm per ml are stated.

It is clear that the particle formation is suppressed according to the invention by using sodium-free potassium phosphate buffers (KP). This effect can be increased by adding a nonionic detergent.

Table 1

MAB L-selectin in buffer	C _{prot} [mg/ml]	particles/ml without stress 2-400 μm	Particles/ml 6 x freezing/thawing 2-400 μm
10 mM KP, 150 mM NaCl, pH 7.2	21.40	875	6245
100 mM KP, 0.01 % by weight Tween80, pH 7.2	18.50	276	332

MAB HBV in buffer	C _{prot} [mg/ml]	particles/ml without stress 2-400 μm	Particles/ml 6 x freezing/thawing 2-400 μm
10 mM KP, 30 mM NaCl, pH 6.6	17.85	544	19085
100 mM KP, 0.01 % by weight Tween80, pH 7.2	18.30	740	695

MAB PDGF-R in buffer	C _{prot} [mg/ml]	particles/ml without stress 2-400 μm	Particles/ml 6 x freezing/thawing 2-400 μm
10 mM KP, 150 mM NaCl, pH 7.2	1.70	130	33795
50 mM KP, 0.01 % by weight Tween80, pH 7.2	1.70	691	677

5

MAB LNGF-R in buffer	C _{prot} [mg/ml]	particles/ml without stress 2-400 μm	Particles/ml 6 x freezing/thawing 2-400 μm
10 mM KP, 150 mM NaCl, pH 7.2	1.70	690	28915
50 mM KP, 0.01 % by weight Tween80, pH 7.2	1.70	1164	1257

List of references

- DE 26 52 636
EP-A 0 018 609
5 EP-A 0 025 275
EP-A 0 314 095
EP-A 0 315 968
EP-A 0 318 081
EP-A 0 599 344
10 GB 8514349
Mendoza, J.A. Biotechnol. Tech. 10 (1991) 535 - 540
US Patent 4,808,705
WO 91/15509
WO 93/22335



The claims defining the invention are as follows:

1. Improved process for preventing the formation of protein aggregates of more than 6000 particles in the range of above 10 μ m and 600 particles in the range of 25 μ m per injection dose in a reconstituted lyophilisate of a pharmaceutical composition of a protein, in which process an aqueous buffered solution of the protein is frozen, thawed, divided into compartments of injectable amounts and these compartments are lyophilized, wherein the aqueous buffered solution of the protein contains potassium phosphate buffer as a buffer substance and as a substance to avoid protein aggregates and the ratio of potassium to sodium ions in the solution is 10:1 or higher.

2. The process claimed in claim 1, wherein the buffer concentration in the compartment is between 10 mmol/l and 300 mmol/l.

3. The process as claimed in claim 1 or claim 2, wherein the osmolarity of the reconstituted solution of the compartment is between 100 and 500 mOsm.

4. The process as claimed in claim 3 wherein the osmolarity of the reconstituted solution of the compartment is 300 \pm 50mOsm.

5. The process as claimed in any one of claims 1-4, wherein the aqueous buffered solution is buffered in the pH range between 6-8.

6. The process as claimed in any one of claims 1-5, wherein the aqueous buffered solution contains a nonionic detergent.

7. The process as claimed in any one of claims 1-6, wherein the aqueous buffered solution contains a sugar at a concentration of 10-100 mg/ml.

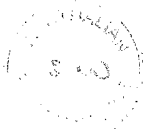
8. The process as claimed in any one of claims 1-7, wherein the protein is an antibody.

9. Meltable solid storage form of an injection dose of a protein containing as protein aggregates a maximum of 6000 particles in the range above 10 μ m and 600 particles in the range of 25 μ m per injection dose, wherein it is essentially amorphous, contains a frozen solution of the protein with potassium phosphate buffer as the only buffer substance and as the only substance to avoid protein aggregates and the ratio of potassium ions to sodium ions is at least 10:1.

10. Solid storage form of an injection dose of a protein as claimed in claim 9, wherein the storage form is prepared by means of lyophilization.

11. Storage form of an injection dose of a protein as claimed in claim 9 or 10, wherein the protein is an antibody.

12. Pharmaceutical composition of a protein in an aqueous buffered solution in the pH range between 6 and 8, wherein the solution contains potassium phosphate buffer



as the only buffer substance and as the only substance to avoid formation of protein aggregates of more than 6000 particles in the range of 10µm and 600 particles in the range of 25µm per injection dose in the reconstituted lyophilisate of said pharmaceutical composition and

- 5 a) the ratio of potassium to sodium ions in the solution is 10:1 or higher,
 b) the buffer concentration is between 10 and 300 mmol/l.
13. Pharmaceutical composition as claimed in claim 12, wherein the protein is an antibody.

10 14. Process for preventing the formation of protein aggregates of more than 6000 particles in the range of above 10µm and 600 particles in the range of 25µm per injection dose in a reconstituted lyophilisate of a pharmaceutical composition of a protein, which process is substantially as hereinbefore described with reference to the Examples.



15. Meltable solid storage form of an injection dose of a protein containing as protein aggregates a maximum of 6000 particles in the range above 10µm and 600 particles in the range of 25µm per injection dose wherein it is essentially amorphous, contains a frozen solution of the protein with potassium phosphate buffer as the only buffer substance and as the only substance to avoid protein aggregates, substantially as hereinbefore described with reference to the Examples.



16. Pharmaceutical composition of a protein in an aqueous buffered solution in the pH range between 6 and 8, wherein the solution contains potassium phosphate buffer as the only buffer substance and as the only substance to avoid formation of protein aggregates of more than 6000 particles in the range of 10µm and 600 particles in the range of 25µm per injection dose in the reconstituted lyophilisate of said pharmaceutical composition, substantially as hereinbefore described with reference to the Examples.

17. A reconstituted lyophilisate of a pharmaceutical composition of a protein, which lyophilisate is prepared according to the process of any one of claims 1-8 or 14.

Dated 14 October, 1999
Boehringer Mannheim GmbH

Patent Attorneys for the Applicant/Nominated Person
SPRUSON & FERGUSON

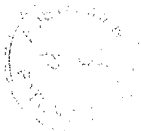


Fig. 1

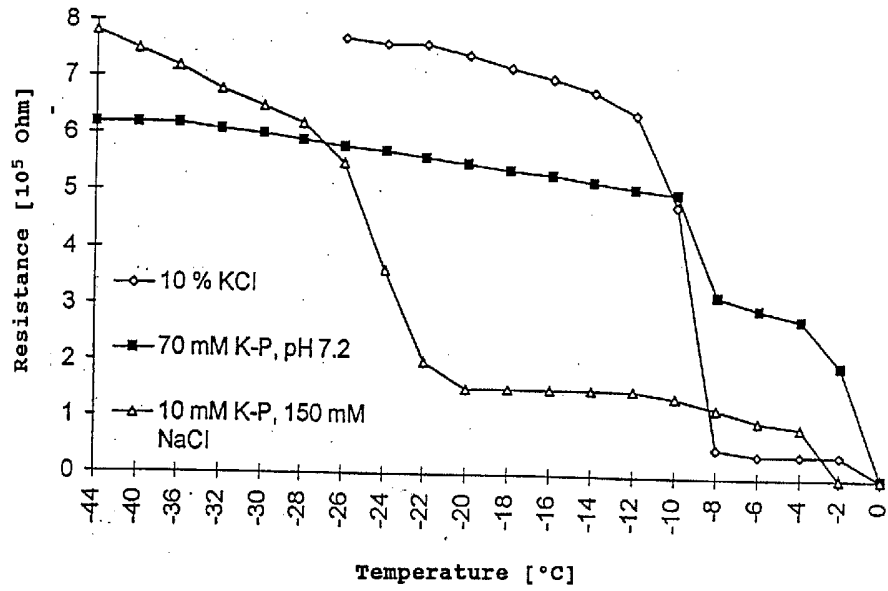


Fig. 2

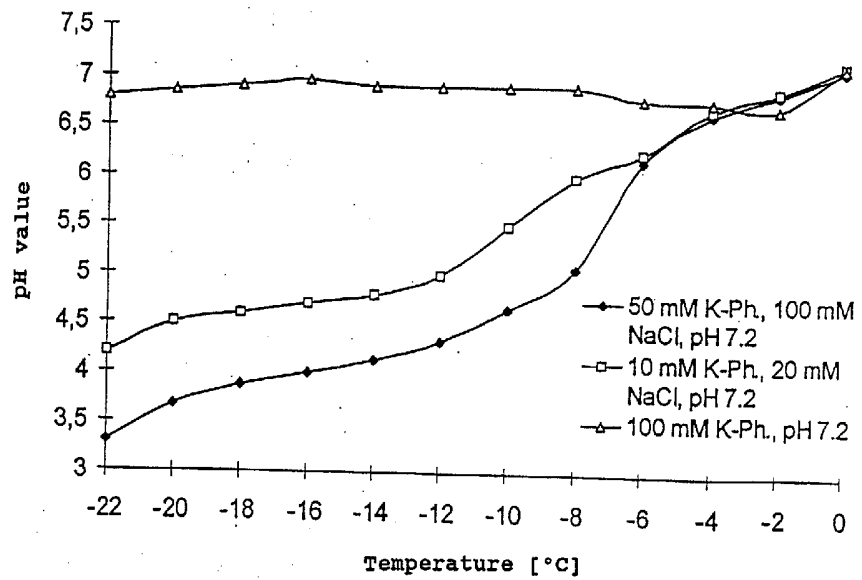




Fig. 3

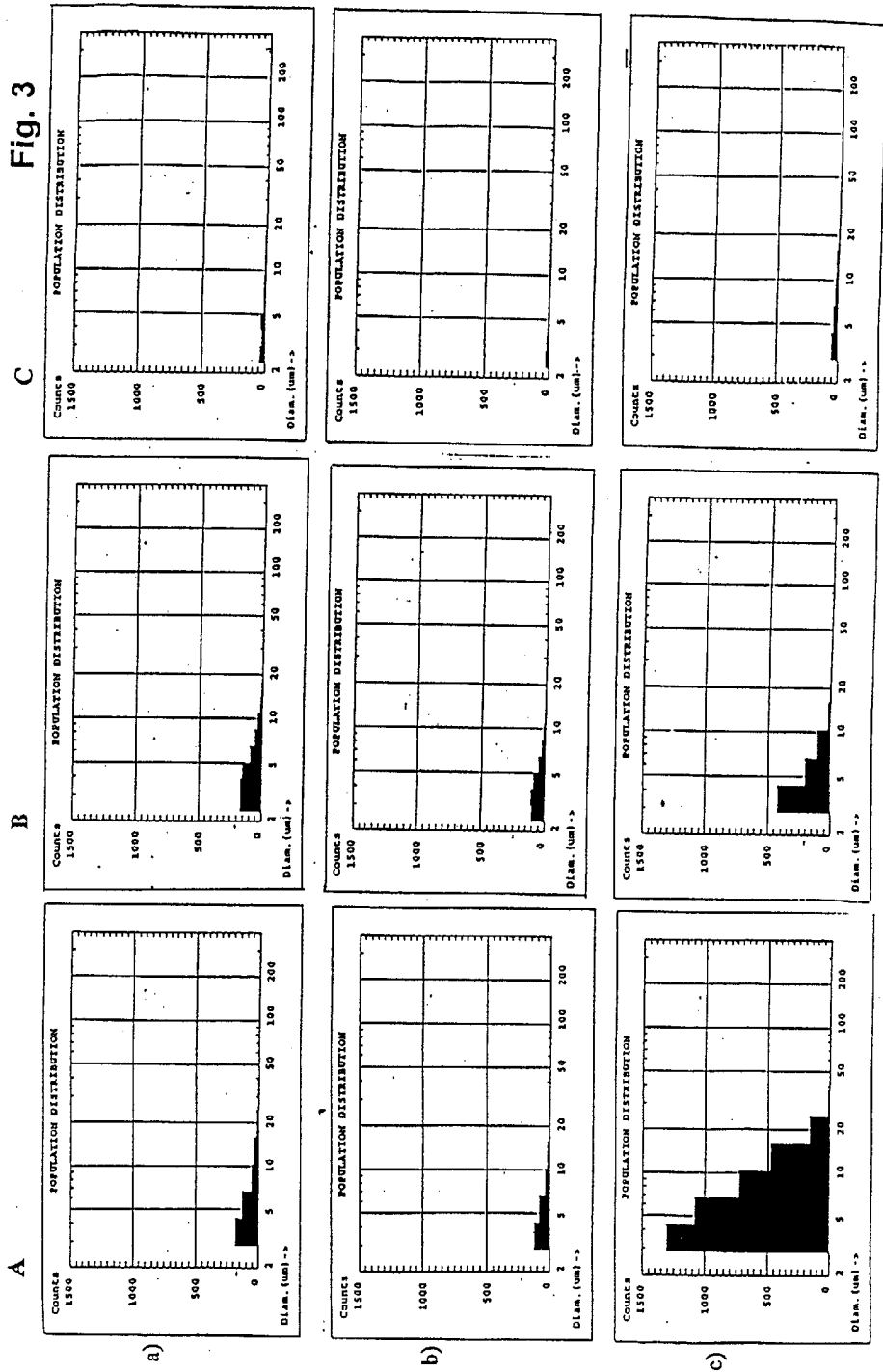




Fig. 4

C.

B.

A.

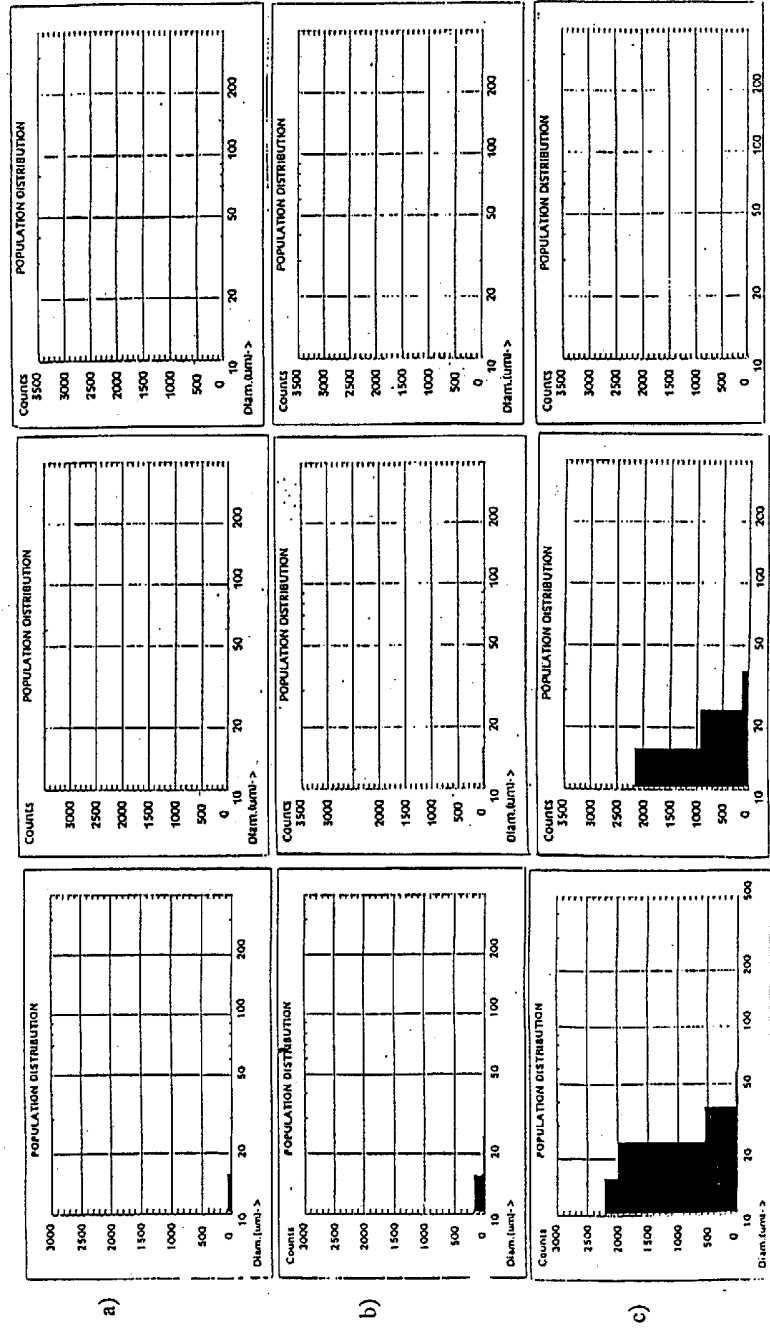
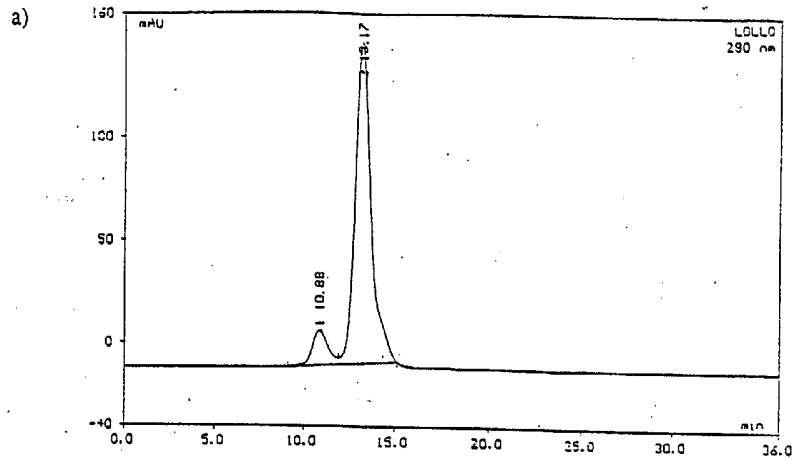


Fig. 5

A



B

