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(54) Title: ROTAVIRUS VACCINE FORMULATIONS		
(57) Abstract <p>The present invention provides novel formulations of rotaviruses useful as vaccines and methods for their preparation.</p>		

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TITLE OF THE INVENTION

ROTAVIRUS VACCINE FORMULATIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

5 Not applicable.

STATEMENT REGARDING FEDERALLY-SPONSORED R&D

Not applicable.

10 REFERENCE TO A MICROFICHE APPENDIX

Not applicable

FIELD OF THE INVENTION

15 The present invention is related to novel liquid and lyophilized formulations of rotaviruses useful as vaccines and methods for their preparation.

BACKGROUND OF THE INVENTION

20 Rotaviruses (RV) cause acute gastroenteritis, a disease that requires hospitalization of infants and young children in developed countries, and a frequent cause of death in children less than 5 years of age in developing regions of the world. Studies in the United States, Australia, and Japan have demonstrated that between 34 and 63% of hospitalizations of children for acute diarrheal disease are associated
25 with rotavirus infection. The incidence of hospitalization for rotavirus gastroenteritis in a health maintenance organization in the U.S. was estimated to be 222 per 100,000 in children from 13 to 24 months of age, and 362 per 100,000 in those less than one year. Infection with rotavirus was associated with 63% of all hospitalizations for acute diarrhea in this
30 pediatric population. A review of mortality data in the U.S. from 1973 to 1983 indicated that 500 deaths per year occur in children less than 4 years old due to diarrheal diseases, and that 20 to 80% of excess winter deaths due to diarrhea in the U.S. are associated with rotavirus infections. Rotaviruses are also responsible for substantial proportion of
35 the mortality associated with diarrheal diseases in third world countries. An effective rotavirus vaccine would therefore have a major impact on the health of children in both the developed and developing areas of the world.

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Rotaviruses have an inner and outer capsid with a double-stranded RNA genome formed by eleven gene segments. Multiple serotypes have been defined by plaque reduction neutralization tests, and studies of reassortant viruses have demonstrated that two outer capsid proteins, VP7 and VP4, are the determinants of virus serotype. The VP7 protein is coded for by either gene segment 7, gene segment 8 or gene segment 9 of a particular human rotavirus. The location of the VP7 encoding gene may be determined for each specific rotavirus by conventional experimental methods. The VP4 protein is an 88,000 dalton major surface structural protein product of gene 4 of a rotavirus. Like VP7, it functions as a major serotype-specific antigen, operative in serum neutralization (SN) tests, capable of inducing serotype-specific neutralizing antibody, and capable in a mouse system of inducing serotype-specific immune protection against rotavirus disease. In some earlier references, the VP4 was referred to as VP3. After 1988, a change in nomenclature, resulted in the more proper reference to this protein as VP4.

Since the gene segments encoding the VP7 and VP4 proteins segregate independently, it has been proposed that serotyping nomenclature include both the G type, determined by VP7, and the P type, determined by VP4. Most human rotavirus infections in the U.S. are caused by viruses of G types 1, 2, 3, or 4, and P types 1, 2, or 3. However, other human rotavirus types, including for example, type G9, are more prevalent in Asia, Europe and certain third world countries.

A number of animal rotaviruses are attenuated in humans, and have been evaluated as potential live rotavirus vaccines, including the bovine serotype G6 WC3 rotavirus. The WC3 vaccine virus was shown to be immunogenic and non-reactogenic in infants, but was inconsistent in providing protective immunity against human rotavirus infection. It has been suggested that serotype-specific immunity is necessary to include consistent protection against rotavirus diarrhea.

There exists a need to the art for effective vaccines providing protective immunity against rotavirus infection and the severe clinical symptoms associated therewith.

For worldwide distribution of rotavirus vaccines, it is necessary to formulate vaccines such that they are stable under a variety of environmental conditions. Components used to stabilize vaccines are

known. However, particular formulations of components useful to stabilize rotavirus vaccines must be determined experimentally. One object of the present invention is present formulations which stabilize rotavirus vaccines.

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SUMMARY OF THE INVENTION

The present invention provides novel formulations of rotaviruses useful as vaccines and methods for their preparation.

10 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Effect of buffer combinations on rotavirus stability at 37°C for 1 week. Data for the G1 reassortant are shown in panel A and the P1 reassortant in panel B. All values are expressed as pfu/mL normalized to the reference, or 0 day, sample. The buffer combinations are represented as follows: 0.05 M sodium citrate + 0.15 M sodium bicarbonate (□), 0.05 M sodium citrate + 0.15 M sodium phosphate (○), 0.05 M lactic acid + 0.15 M sodium bicarbonate (Δ), 0.05 M lactic acid + 0.15 M sodium phosphate (∇) and 0.20 M sodium succinate + 0.05 M sodium phosphate (◇). All formulations have pH values of 7.

Figure 2. Acid neutralizing ability of formulation buffers compared to bicarbonate. One mL of each buffer was titrated with 0.01 N HCl. Symbols: 0.4 M sodium bicarbonate (●), 0.1 M sodium citrate + 0.3 M sodium phosphate (○), 0.1 M sodium citrate + 0.3 M sodium bicarbonate (+), and 0.2 M sodium succinate + 0.1 M sodium phosphate (∇).

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Figure 3. Stability data for reassortant rotavirus in liquid formulations of 5% sucrose/0.1 M sodium succinate/0.05 M sodium phosphate after storage at various temperatures. Data for G1 rotavirus is shown in panel A and for P1 rotavirus in panel B.

Figure 4. Stability data for reassortant rotavirus in liquid formulations of 50% sucrose/0.1 M sodium succinate/0.05 M sodium phosphate after storage at various temperatures. Data for G1 rotavirus is shown in panel A and for P1 rotavirus in panel B.

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Figure 5. Stability data for G1 rotavirus liquid formulations with higher buffer, sucrose, and hydrolyzed gelatin concentrations at various temperatures. Panel A shows data for G1 rotavirus in Williams' E media ("WE"), 50% sucrose, 0.2 M sodium succinate, and 0.1

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M sodium phosphate. Stability data for vaccine in Williams' E media, 70% sucrose, 0.2 M sodium succinate, and 0.1 M sodium phosphate is shown in panel B. Panel C shows data for G1 rotavirus in 50% sucrose, 0.1 M sodium citrate, and 0.3 M sodium phosphate; panel D shows data for G1 rotavirus in Williams' E media, 50% sucrose, 0.2 M sodium succinate, 0.1 M sodium phosphate, and 5% hydrolyzed gelatin.

Figure 6. Stability data for P1 rotavirus liquid formulations with higher buffer, sucrose, and hydrolyzed gelatin concentrations at various temperatures. Panel A shows data for P1 rotavirus in Williams' E media, 50% sucrose, 0.2 M sodium succinate, and 0.1 M sodium phosphate. Stability data for vaccine in Williams' E media, 70% sucrose, 0.2 M sodium succinate, and 0.1 M sodium phosphate is shown in panel B. Panel C shows data for P1 rotavirus in 50% sucrose, 0.1 M sodium citrate, and 0.3 M sodium phosphate; panel D shows data for P1 rotavirus in Williams' E media, 50% sucrose, 0.2 M sodium succinate, 0.1 M sodium phosphate, and 5% hydrolyzed gelatin.

Figure 7. Stability data for rotavirus liquid formulations in 50% sucrose, 0.1 M sodium succinate, and 0.05 M sodium phosphate after storage at various temperatures. Data for G2 rotavirus is shown in panel A and for G3 in panel B.

Figure 8. Stability data for G1 rotavirus lyophilized formulations after storage at various temperatures. Panel A shows data for G1 rotavirus dialyzed prior to lyophilization into 1% sucrose, 4% mannitol, and 10 mM sodium phosphate. Stability data for vaccine dialyzed prior to lyophilization into 1% lactose, 4% mannitol, and 10 mM sodium phosphate is shown in panel B. Panel C shows data for G1 rotavirus diluted phosphate prior to lyophilization into 1% sucrose, 4% mannitol, and 75 mM sodium phosphate.

Figure 9. Stability data for P1 rotavirus lyophilized formulations after storage at various temperatures. Panel A shows data for P1 rotavirus dialyzed prior to lyophilization into 1% sucrose, 4% mannitol, and 10 mM sodium phosphate. Stability data for vaccine dialyzed prior to lyophilization into 1% lactose, 4% mannitol, and 10 mM sodium phosphate is shown in panel B. Panel C shows data for P1 rotavirus diluted prior to lyophilization into 1% sucrose, 4% mannitol, and 75 mM sodium phosphate.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides novel formulations of rotaviruses useful as vaccines and methods for their preparation. More particularly, the invention relates to stabilizing formulations for liquid and lyophilized rotavirus vaccines. In addition, some of these formulations can be orally delivered either with or without preneutralization of gastric acid since some of the formulations contain high levels of buffering components.

Due to the worldwide distribution of vaccines and the diversity of ambient temperatures, it is necessary to formulate vaccines such that they are stable under a variety of environmental conditions. A variety of stabilization methods have been used. These include the following:

- a) Low temperatures (-10°C to -70°C). Most vaccines are stable during storage at extremely low temperatures. However, low temperature storage facilities are costly and are not always available; this limits the utility and practicality of this approach.
- b) Lyophilization. Freeze-dried vaccines are reasonably stable and can be stored at 2-8°C for a predefined length of time. Lyophilization may, however, result in a loss of viral titer during drying thereby reducing the yield of the manufacturing process. In addition, during long-term storage, a lyophilized vaccine may still deteriorate, to the point where it may or does not have sufficient titer to confer immunization. Furthermore, since a lyophilized vaccine requires reconstitution prior to use, a liquid reconstituted preparation may lose potency while standing at room temperature before use. This loss of titer during reconstitution may also result in insufficient titer to confer immunity.
- c) Stabilizers. These are specific chemical compounds that interact and stabilize biological molecules and/or general pharmaceutical excipients that are added to the vaccine and are used in conjunction with either lower temperature storage or lyophilization methods.

These formulations can be prepared by either (1) dilution of bulk vaccine into the stabilizer, (2) dialysis/diafiltration into the stabilizer, or (3) concentration of bulk vaccine and diafiltration into the stabilizer, followed by lyophilization if required.

The stabilizer composition of the present invention contains the following ingredients in about the amounts indicated. For convenience the amounts are stated round numbers. However, one skilled in the art will recognize that amounts within 10 or 20 percent of the stated values can also be expected to be appropriate, *i.e.*, where 20% is stated, a range of from 16-18% to 22-24% is implicit and can be appropriate. For liquid formulations:

	Sucrose:	1-70% (w/v)
	Sodium or potassium phosphate:	0.01 - 2 M
10	Sodium succinate or sodium citrate:	0.05 - 2 M
	Tissue culture medium, saline, or water:	0-balance of remaining volume

For lyophilized formulations:

	Sodium phosphate	0.05 - 2 M
15	Sucrose	1 - 20% (w/v)
	Mannitol	1 - 20% (w/v)
	Lactose	1 - 20% (w/v)

In addition, the following can also be present:

	Hydrolyzed gelatin	2.5% (w/v)
20	Sodium chloride	150 mM
	Sodium glutamate	7 mM

The following compounds can be used in place of sucrose, and at comparable osmolality: fucose, trehalose, polyaspartic acid, inositol hexaphosphate (phytic acid), sialic acid or N-acetylneuraminic acid-lactose. Also, any suitable sugar or sugar alcohol such as dextrose, mannitol, lactose, or sorbitol, can be substituted for sucrose at concentrations effective in achieving the desired stabilization.

The concentration of sugar relates to the viscosity of the formulation. In instances where reduced viscosity is desired, it is known in the art to be preferable to use lower concentrations of sugar, *e.g.*, sucrose. It will also be appreciated by persons in the art that the upper limit for the concentration of sugar can be dictated by the ability of a formulation to undergo required filtration or processing steps.

Tissue culture medium, saline or water can be used as a diluent. Frequently, Williams' E medium ("WE") is used, by which we mean either Williams' E medium or Williams' medium E modified.

Also, buffering agents to neutralize gastric acid are not limited to citrate, phosphate and succinate and could include bicarbonate or common carboxylic acids (carboxylates) such as, but not limited to, fumarate, tartarate, lactate, maleate, etc. The
5 appropriateness of any of these can be assessed by simply trying a formulation in which these agents are substituted or combined with phosphate, citrate or succinate. Up to about 2.0 M carboxylates can be used in the liquid and lyophilized formulations of this invention, however, we prefer to use less than about 1.0 M, *e.g.*, about 0.05 - 0.9 M,
10 and can be less than about 0.7 M, *e.g.*, 0.05 to about 0.7 M. It is also preferable to use less than 0.5 M, *e.g.*, about 0.05 to 0.45 M. Particular concentrations in these ranges can be appropriate. Also, higher concentrations of buffering components (*e.g.* phosphate, succinate, citrate) can be used if, for example, additional gastric neutralization is
15 required. In instances where additional buffering capacity is useful in phosphate/citrate or phosphate/succinate buffers, it is preferable to further increase the concentrations of succinate or citrate as the buffering agent rather than phosphates.

Up to about 2.0 M phosphate can be used in the liquid and
20 lyophilized formulations of this invention, however, we prefer to use less than about 1.0 M, *e.g.*, about 0.010 - 0.8 M, and often less than 0.5 M, *e.g.*, about 0.010 to 0.45 M. It is most preferable to use less than about 0.35 M, *e.g.*, 0.010 - 0.30 M. Particular concentrations in these ranges can be appropriate. In liquid formulations, we prefer to maintain the
25 concentration of phosphate about or below 0.30 M, *e.g.*, 0.010 - 0.35 M to avoid the precipitation of phosphate salts, *e.g.*, during long term storage or freeze/thaw cycles. Thus, the upper limit for the concentration of phosphate in any particular formulation can be dictated by the formation or precipitation of phosphate salts and whether the salts negatively affect
30 the performance of the formulation in areas such as stability and administration. Particular concentrations can be readily determined for any particular formulation by standard empirical testing including pH adjustments in the range of pH 6-8.

For general guidance, examples of the acid neutralizing
35 capacities of some liquid formulations are presented in Table 1 below. Also provided are some preferred formulations.

Table 1.

Acid-neutralizing capacities (ANC) of rotavirus stabilizer formulations.

Sodium Phosphate (M)	Sodium Citrate (M)	Sucrose (%)	ANC (mEq/mL)
0.30	0.10	50	0.48
0.30	0.70	50	1.55
0.75	0.25	50	1.07

5 For lyophilized formulations:

Sodium phosphate 20 mM

Hydrolyzed gelatin 2.5% (w/v)

Sucrose 5% (w/v)

Sodium chloride 150 mM

10 Sodium glutamate 7 mM

or

Sucrose or Lactose 1% (w/v)

15 Mannitol 4% (w/v)

Sodium or potassium phosphate 0.01-0.1 M

A preferred formulation of the liquid viral vaccine stabilizer
of the present invention is as follows:

20 Sucrose 50% (w/v)

Sodium or potassium phosphate 0.1 M

Sodium succinate 0.2 M

Tissue culture medium used for all dilutions

25 or

Sucrose 50% (w/v)

Sodium or potassium phosphate 0.3 M

Sodium citrate 0.1 M

30 Tissue culture medium used for all dilutions

or

	Sucrose	30% (w/v)
	Sodium or potassium phosphate	0.3 M
5	Sodium citrate	0.7 M
	Tissue culture medium	used for all dilutions

In these preferred formulations, it can be appropriate to use saline or water in place of, or in combination with, the tissue culture medium.

10 This invention involves formulations of reassortant rotaviruses (RRV) suitable for use as vaccines, which are characterized by safety to humans and the ability to confer immune protection against human rotavirus infection. The RRV are produced by genetic
 15 reassortment between an attenuated bovine rotavirus (preferably WC3 or progeny thereof) and at least one rotavirus representing an epidemiologically important human serotype. In one type of RRV, the human rotavirus contributes to the reassortant at least the gene
 20 segment encoding the VP7 protein. In another type of RRV, the human rotavirus parent contributes to the reassortant at least the gene segment encoding the VP4 protein. In still another type of RRV, the human rotavirus parental strain contributes at least both the VP7 and VP4 gene
 segments. In additional types of RRV, the human rotavirus parental strain may contribute gene segments in addition to those which encode the VP7 and/or VP4 antigens.

25 The human rotavirus gene which encodes for the neutralization antigen VP7 and/or VP4 in the RRV may be selected from any human rotavirus serotype for which immunization is desired. Desirably, in a reassortant of this invention the VP7 gene is derived from a G1, G2, G3, or G4 human rotavirus serotype and the VP4 protein is
 30 derived from a human P1 or P2 serotype. Among the rotavirus strains noted to be clinically significant in human rotavirus infections (hereinafter "human rotavirus strains"), including strains useful in the present invention, are the strains provided below:

- 35 serotype G1: WI79, Wa, D;
 serotype G2: strains WISC2 and DS1;
 serotype G3: strains WI78, P, HCR3A;
 serotype G4: Bricout (Br) B, ST3;

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serotype G8: 69M;
serotype G9: WI61;
serotype P1: WI79, WI78, WI61, Wa;
serotype P2: DS1; and
5 serotype P3: WISC2, BrB, BrA, M37.

10 This list of human rotavirus strains is non-exclusive. For example, several rotavirus strains previously identified in animal infections have also been found in human infections. These strains can be anticipated to be useful as 'human' rotavirus strains for the purposes of this invention, *e.g.*, the 'porcine' rotavirus OSU, a serotype G5, and the 'bovine' rotavirus B223, a serotype G10. One of skill in the art can readily obtain other appropriate human strains from suitable depositories or academic or commercial sources.

15 The non-human genes present in the reassortants of this invention are obtained preferably from the attenuated, serotype G6, bovine rotavirus strain WC3 or its progeny, described in detail in U.S. Patent 4,636,385. However, other rotavirus reassortants, particularly other bovine reassortants, are also preferred.

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Table 2

Human Serotype	Parent or Reassortant	ATCC#	Deposit Date
G1	WI79-3,9 ^a	VR2194	Nov. 25, 1987
		VR2196	Nov. 25, 1987
	WI79-4,9	VR2415	July 8, 1993
G2	WI79-3+WISC2-9		Dec. 7, 1994
	WISC2 parental strain	VR2417	July 8, 1993
G3	WI78-8		Dec. 7, 1994
	WI78-1,6-11	VR2195	Nov. 25, 1987
	WI78-1,7-11 ^b		
G4	Bricout B-9		Dec. 7, 1994
P1	WI79-4	VR2377	June 19, 1992
	WI79-4,9	VR2415	July 8, 1993
	WI61-4 ^b		
P2	DS1-4 ^b		

^aOriginally named WI79-9. The two deposits represent different passage levels of the reassortant.

5 ^bNot deposited.

10 The deposits of WI79-3,9 and WI78-1,6-11 have been converted to comply with requirements of the Budapest Treaty. All other deposits have been originally made under the Budapest Treaty. All
15 restrictions on the availability to the public of the deposited material identified in Table 2 will be irrevocably removed upon the grant of a patent on this application, the culture(s) will be maintained for a period of 30 years from the deposit date, or at least five years after the most recent request for a sample, whichever is longer; and the deposit will be replaced if viable samples cannot be dispensed by the depository. During

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the pendency of this patent application, access to these deposits will be afforded to one determined by the Commissioner to be entitled thereto.

Vaccine Compositions

- 5 Vaccines for providing immunological protection against acute diarrhea caused by human rotavirus infection can contain one or more rotavirus reassortants in a formulation of the present invention. Exemplary rotavirus reassortants and combinations thereof and their use in vaccines are found in U.S. Patent 5,626,851 and in U.S. Application Ser. No. 08/456,906, both of which are incorporated herein by 10 references in their entireties. Several exemplary vaccine compositions are summarized in Table 3.

Table 3

15

Vaccine compositions	Preferred Reassortants
G1 + G2 + G3 + G4	WI79-3,9 + (WI79-3+WISC2) + WI78-8 + BrB-9
G1 + G2 + G3 + G4 + P1	WI79-3,9 + (WI79-3+WISC2-9) + WI78-8 + BrB-9 + WI79-4
G1 + G2 + G3 + P1	WI79-3,9 + (WI79-3+WISC2-9) + WI78-8 + WI79-4
G1 + P1	WI79-3,9 + WI79-4
G1 + G2 + G3	WI79-3,9 + (WI79-3+WISC2-9) + WI78-8
G1 + G2 + G3 + G4 + P1 + P2	WI79-3,9 + (WI79-3+WISC2-9) + WI78-8 + BrB-9 + WI79-4 + DS1-4
G1	WI79-3,9

The rotavirus vaccines of the invention can contain conventional components. Suitable components are known to those of skill in the art. These vaccine compositions can be prepared in liquid forms or in lyophilized forms. Other optional components, *e.g.*,
5 stabilizers, buffers, preservatives, flavorings, excipients and the like, can be added. The determination of specific formulations useful in stabilizing vaccine compositions has required extensive experimentation.

When adapted for oral administration, one formulation
10 includes as a carrier Williams' E medium ("WE")/50% sucrose/0.1 M succinate/50 mM phosphate liquid. Other formulations include 0.2 M succinate and 0.1 M phosphate, or 0.1 M citrate and 0.3 M phosphate. Another formulation includes 0.7M citrate and 0.3M phosphate with
15 Williams' E medium/30% sucrose. In addition, novel adjuvants to boost or augment immune responses developed for oral administration should be compatible with these formulations. When adapted for parenteral administration, conventional adjuvants (*e.g.*, aluminum salts) or novel adjuvants can also be employed in the vaccine composition.

Optionally, the vaccine may preferably be formulated to
20 contain other active ingredients and/or immunizing antigens. For example, when adapted for oral administration, formulation with the Sabin polio vaccine may be desirable.

The dosage regimen involved in a method for vaccination, including the timing, number and amounts of booster vaccines, will be
25 determined considering various hosts and environmental factors, *e.g.*, the age of the patients time of administration and the geographical location and environment.

Method of Vaccination

30 Therefore, also included in the invention is a method of vaccinating humans against human rotavirus infection with the novel RRV vaccine compositions. The vaccine compositions including one or more of the reassortants described herein are administered, preferably by the oral route, in a suitable dose, preferably liquid.

35 The dosage for all routes of administration is generally between 10^5 and 10^9 plaque forming units (pfu) of the reassortant with the preferred dosage being 10^7 pfu. Additional doses of the vaccines can

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be also be administered. It may be preferable to inoculate susceptible infants and children on an annual basis prior to the "rotavirus season". Rotavirus infection in humans has been observed to occur in various geographical regions during the same season, *e.g.*, in winter in the United States. Repeated inoculations prior to that season for susceptible infants and children may be indicated. For example, one currently preferred dosage regimen for the U.S. includes three doses approximately two months apart prior to the beginning of the rotavirus season.

10 The following examples illustrate methods for preparing the RRV vaccine formulations of the invention. These examples are illustrative only and do not limit the scope of the invention.

EXAMPLE 1

15 Administration of a vaccine by the oral route exposes the vaccine to the low pH gastric environment. Most vaccines tend to be inactivated by such extreme conditions. In order to ensure delivery of active vaccine, potential buffers were examined for acid neutralizing capacity as well as their ability to stabilize rotavirus.

20

Rotavirus Stability in the presence of Acid Neutralizing Buffers

 Citrate, lactate, and succinate buffer combinations (5 total) were evaluated for their effect on rotavirus stability at 37°C over a 1 week period. The buffers, whose concentrations are given in the legend to Figure 1, were added to an equal volume of rotavirus in WE medium and incubated for 0, 3, or 7 days.

 For the G1 serotype, the bicarbonate combinations had no effect on the time to lose one half of the infectious titer ($t_{1/2}$) since the values were similar to those in 5% sucrose (0.5 days). In contrast, the phosphate buffers containing citrate, lactate, and succinate stabilized the virus exhibiting $t_{1/2}$ values of 1.2, 1.4, and 1.5 days, respectively (Figure 1).

 As shown in Figure 1, phosphate had a similar effect on the stability of P1. The lactate/phosphate buffer had a $t_{1/2}$ of 2.4 days, and the succinate/phosphate combination had a $t_{1/2}$ of 6.8 days compared to a value of ca. 1.2 days for a 5% sucrose solution. Similar to their effect on the G1 rotavirus, the buffer combinations containing bicarbonate

conferred less stability on the P1 serotype than similar buffers containing phosphate.

5 Combination of Rotavirus with Acid Neutralizing Buffer - Potential Single Administration

The stabilizing effect of succinate/phosphate as well as other buffers suggests that the formulation can contain an acid neutralizer. One mL of the buffers tested appear to neutralize enough acid to keep the pH above 3.5 (Figure 2) which is known from our direct
 10 experimentation and the scientific literature to be necessary for preservation of rotavirus infectivity. Based on infant gastric acid volumes and acid secretion rates, the pH can be maintained in vivo for approximately 0.5 h with the liquid formulations to be described in this work, however, human clinical studies will have to be performed to
 15 confirm these assumptions. As another test of buffering ability, the USP test for acid-neutralizing capacity was performed. As shown in Table 4, RV formulation buffering components are more effective than an equal volume of infant formula.

20 **Table 4.** Acid-neutralizing capacity as measured by USP test for a novel liquid rotavirus formulation (1); formulation used in previous clinical trials by others (2); tissue culture media (3); infant formula (4), and an antacid (5).

	<u>mEq/mL</u>
25 (1) 50% sucrose + 0.2 M sodium succinate + 0.1 M sodium phosphate in Williams' E media ("WE")	0.41
30 (2) 0.3 M sodium bicarbonate + 0.033 M sodium citrate	0.40
(3) Williams' E media	0.02
(4) Isomil®	0.12
35 (5) Mylanta®	5.17

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For lyophilized formulations, additional buffering capacity can be attained by reconstitution with an acid-neutralizing buffer described in this work or commonly available acid neutralizing compounds such as a bicarbonate solution. Thus, with either a liquid or lyophilized formulation, adequate buffering capacity is possible without pretreatment. Consequently, the rotavirus vaccine may preferably be able to be administered in a single administration rather than with a separate gastric neutralization step followed by the vaccine. However, simultaneous administration of buffer and vaccine will have to be further evaluated in patients to have confidence in the efficacy of the vaccine using in this approach. If pretreatment of patients (formula feeding or dose of bicarbonate or an antacid such as Mylanta®) is still necessary to ensure adequate gastric acid neutralization for routine oral vaccination with rotavirus, these formulations will still provide a large enhancement in the storage stability as described in the next section. Furthermore, the rotavirus reassortants are compatible with infant formulae (e.g., Isomil® and Similac®) as well as bicarbonate buffers and show comparable thermal stability in the presence or absence of these neutralizers.

EXAMPLE 2

Putative binding sites on rotavirus can be considered as targets for stabilization. Calcium and zinc binding sites have been suggested to be present in rotavirus proteins and the presence of these cations may stabilize the vaccine. Other divalent cations may also bind to these or other sites and stabilize rotavirus and its reassortants. Binding by other compounds was also investigated in order to identify compounds that can stabilize the vaccine yet not interfere with its ability to confer immunogenicity.

a. Effect of Divalent Metal Ions

The addition of metal chelators such as EDTA or EGTA is known to cause a loss in RV infectivity, presumably by disrupting the outer shell of the RV. This suggests that metals may be necessary for structural integrity. Accordingly, divalent metal ions were examined to assess their potential ability to stabilize rotavirus (RV).

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Rotavirus in WE medium was dialyzed at 4°C for approximately 16 hours in 20 mM Tris buffer and 100 mM NaCl. The final solution was supplemented with 10 mM of either CaCl₂, MnCl₂, MgCl₂, ZnCl₂, or CaCl₂ + ZnCl₂ to yield a final concentration of 10 mM metal ion. The samples can be filtered prior to formulation. Samples were incubated at 37°C for 0, 2/3, and 7 days and were then stored at -70°C until assayed. Each data point represents an average of 2 replicate samples.

As shown in Table 5, calcium and manganese do improve the stability of both G1 and P1 rotavirus reassortants at 37°C when the formulations are prepared by dialysis of the rotavirus bulks into formulations without tissue culture medium. Zinc dramatically decreased the inactivation half-life ($t_{1/2}$) of G1 and significantly decrease the $t_{1/2}$ of P1 in the presence or absence of calcium. It is possible that Zn²⁺ may be replacing Ca²⁺, causing the destabilization of the outer capsid in a manner analogous to the removal of Ca²⁺ by EDTA. An alternative explanations may be that Zn²⁺ activates endogenous metalloproteinases or potentiates nucleases derived from the cell culture. The addition of divalent metals does not increase the thermal stability of RV when formulated in a stabilizer containing tissue culture medium such as Williams' E or Williams' modified E. The G2 and G3 reassortants appeared to behave similarly to G1 and P1 reassortants when compared in cation-supplemented tissue culture media.

Thus, in preparing stabilized formulations of rotaviruses as described herein, it is preferable that sufficient levels of divalent metal ions be present. These metal ions are most likely provided by the tissue culture medium and cells used in cell culture to prepare the bulk virus. Metal ions can also be supplemented, if necessary, in the final formulation by direct addition individually or through the use of tissue culture medium.

Table 5. Effect of divalent metals on the inactivation kinetics of rotavirus reassortants. Values represent the log loss in viral titer after 3 days at 37°C.

	<u>Cation (10 mM) added</u>	<u>P1</u>	<u>G1</u>
5	none	2.2	2.5
	Ca ²⁺	0.5	0.2
	Zn ²⁺	>3.8	>4.0
10	Zn ²⁺ + Ca ²⁺	>3.9	>3.9
	Mn ²⁺	1.5	2.2
	Mg ²⁺	2.6	4.2

15 **b. Effect of Biologically Relevant Sugars and Polyanions**

Preliminary experiments described above showed rotavirus reassortants are stabilized by phosphate buffer. Since there are examples of monomeric proteins which are stabilized by phosphate that are also stabilized by related polyanions such as sulfate, inositol hexaphosphate (phytic acid) and various sulfated compounds (heparin and sulfated β -cyclodextrin), these compounds were tested for their ability to stabilize rotavirus. Polymeric forms of polyanions are generally more effective stabilizers since a higher charge density can be maintained at lower concentrations of ligand, therefore, polyaspartic acid was also examined due to its high negative charge density. Sialic acid (N-acetylneuraminic acid) was examined since it may bind to VP4 and, therefore, may provide protection from thermally-induced degradation or unfolding. Likewise, sialic acid derivatives such as N-acetylneuraminic acid-lactose and mucin were tested. The loss of RV infectivity with host maturation has been suggested to be due to a switch in the presence of sialic acid to fucose; consequently fucose was examined. Lastly, trehalose was examined because of its reputed properties as a favorable drying excipient.

As can be seen in Table 6, a variety of compounds can be added to rotavirus formulations and stabilize the virus during accelerated stability testing. Inositol hexaphosphate showed the greatest ability to stabilize RV compared to the other ligands in this study. For

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G1, a 4-fold increase in thermal stability at 37°C was observed. Mucin prevents infectivity, probably not by destabilizing the virion structure but rather sequestering RV (clumps were observed prior to assay). The sulfated polymers had a negligible effect, however, all other tested
5 compounds stabilized RV to varying degrees. For example, trehalose extended the inactivation half-life for G1 by greater than 2-fold and P1 by less than 50%.

Sialic acid stabilized both G1 and P1 RV. Sialic acid should stabilize the G types and not the P types if the binding site is located on
10 VP4. In these experiments, P1 appeared to have a lower half-life in the presence of polyanions in general. The lower $t_{1/2}$ in the presence of heparin and poly aspartic acid may suggest that RV bind more tightly to these ligands rather than being destabilized by them. The mechanism of stability suppression is not entirely clear. A low level of infectivity as
15 measured by the plaque assay can be caused by destabilization of the virion itself or sequestration of RV by the ligand. If the association between RV and the excipient is moderate, the ligand would be expected to dissociate under the diluted conditions of the assay (as well as in vivo). Tightly bound complexes can contain stable viral particles, yet are not
20 infectious since they are unable to dissociate. This latter case appears to apply to mucin, heparin, and possibly polyaspartic acid. Also, adverse effects of the excipients on the cells used in the plaque assay cannot be disregarded. Regardless of the mechanism, certain polyanions provide no advantage. Inositol hexaphosphate appears to be the most favorable
25 of all the ligands examined, exceeding the stability induced by phosphate-containing buffers. These results also support previous studies described in this work which show phosphate dramatically stabilizes RV. Thus, a variety of phosphates (*e.g.*, monophosphates and polyphosphates) and phosphorylated compounds (*e.g.*, phosphorylated
30 sugars) can stabilize rotavirus.

Table 6. Effect of polyanions and sugars on the inactivation kinetics. Samples were incubated at 37°C for 1 week.

	added to RV in WE	$t_{1/2}$ (days) for G1	$t_{1/2}$ (days) for P1
5	5% sulfated β -cyclodextrin	0.5	0.8
	5% fucose	1.2	1.7
	5% poly-aspartic acid	1.5	0.6
	1% inositol hexaphosphate	2.0	3.2
10	1% heparin	0.7	<0.1
	1% sialic acid	0.8	1.4
	1% N-acetylneuraminic acid-lactose	1.2	1.5
	1% mucin	<0.1	<0.1
	5% trehalose	1.3	2.0
15	5% sucrose	0.5	1.4

EXAMPLE 3

One-year probe stability data were obtained for several optimized lyophilized and liquid formulations of G1 and P1 rotavirus at various temperatures and compared to the stability data of an unoptimized formulation, WE medium/5% sucrose. Optimized liquid formulations containing rotavirus reassortants in WE medium containing sucrose, sodium phosphate, and sodium succinate or sodium citrate showed a substantial improvement in stability. Further improvements in storage stability were observed for lyophilized formulations. With the appropriate formulation, the thermostability of rotavirus exceeds that of existing live-virus liquid (*i.e.*, OPV) and lyophilized (*e.g.*, measles) vaccines.

The stabilizing effect of either the succinate/phosphate or the citrate/phosphate buffers offers the potential of combining stability enhancement with a gastric neutralization. Liquid formulations as well as lyophilized formulations that can be reconstituted using this buffer can allow the formulation to be delivered in a single administration.

a. Liquid Formulation Stability Data

When formulated in Williams' E medium/5% sucrose/0.1 M succinate/50 mM phosphate at pH 7, the G1 rotavirus reassortant vaccine loses 0.7 log titer after 1 year at 4°C when compared to samples stored at -70°C (Figure 3). The P1 reassortant vaccine loses 0.2 log under the same conditions. After 6 months at 22°C, the G1 reassortant lost 2.6 logs of infectious titer while the P1 reassortant rotavirus lost 5.2 logs. This can be compared to the unoptimized liquid formulation of the G1 reassortant in Williams' E medium/5% sucrose that was recently used in clinical trials which lost greater than 5 logs of infectivity after incubation for 6 months at 22°C and 1-2 logs at 4°C after one year. These data demonstrate the additional stabilizing effect of the specific buffer combinations described in this work.

In Williams' E medium/50% sucrose/0.1 M succinate/50 mM phosphate at pH 7, the G1 rotavirus reassortant vaccine loses 0.8 logs titer after 1 year at 4°C when compared to samples stored at -70°C (Figure 4). The P1 reassortant vaccine loses less than 0.3 logs under the same conditions. At 22°C, both G1 and P1 vaccines lose about 2 logs of infectivity after 1 year. These data demonstrate the additional stabilizing effect of high sugar concentrations.

Additional formulations with higher buffer concentrations (Williams' E medium/50% sucrose/0.2 M succinate/0.1 M phosphate, pH 7) further stabilize the G1 rotavirus vaccine at 4°C resulting in no significant loss of titer when compared to similar samples stored at -70°C (Figure 5). Moreover, no loss in G1 titer is observed for any of the optimized liquid formulations stored at 4°C for one year. The infectivity of the P1 reassortant is 0.2 logs less than the -70°C samples for all formulations (Figure 6). Although the stabilities of both G1 and P1 rotavirus reassortants at 4°C are similar for formulations using higher buffer concentrations, the formulation containing Williams' E medium/50% sucrose/0.1 M citrate/0.3 M phosphate at pH 7 shows less loss at 22°C when compared to other formulations. For example, G1 rotavirus in Williams' E medium/50% sucrose/0.2 M succinate/0.1 M phosphate shows a 1.5 log loss in titer after one year at 22°C, whereas the Williams' E medium/50% sucrose/0.1 M citrate/0.3 M phosphate formulation shows only a 0.6 log loss after this period. The higher phosphate concentration in the latter formulation can be responsible for

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the increased stability since the presence of phosphate and phosphorylated compounds increase the thermostability of rotavirus reassortants as demonstrated by our earlier screening experiments. Although rotavirus in the citrate/phosphate buffered formulation
5 appears to be more stable at 22°C, it is less stable at 45°C for both reassortants and at 37°C for P1 rotavirus.

After 12 months at 4°C in Williams' E medium/50% sucrose/0.1 M succinate/50 mM phosphate at pH 7, the G2 rotavirus reassortant lost 0.2 log of infectivity and the G3 reassortant decreased in
10 titer by 0.3 log when compared to similar samples stored at -70°C (Figure 7). Compared to G1 and P1 reassortants in similar formulations (Figure 3), G2 and G3 have stabilities comparable to that of the P1 rotavirus reassortant and better than that seen with the G1 reassortant at 4°C. However, the G2 and G3 vaccines appear to be less stable than the G1
15 vaccine at 22°C.

The stability of G1 reassortants was studied in the presence and absence of tissue culture medium in formulations including sucrose, phosphate and citrate (Table 7). Formulation A, containing only 5% sucrose in WE, served as the standard in this study. Test
20 formulation B contains 0.3 M sodium phosphate and 0.1 M sodium citrate with 50% sucrose in WE. Test formulation C contains 50% sucrose, 0.3 M sodium phosphate and 0.1 M sodium citrate without WE. The viral bulk is diluted 10-fold into formulations B or C. Thus, 100% of the liquid medium in B is tissue culture medium whereas 10% of the
25 liquid medium in C is tissue culture medium. In C, the viral bulk is the only source of tissue culture medium. As shown in Table 7, formulations B and C showed greater stability than formulation A. The presence or absence of tissue culture medium in the formulations had a small, but measureable, effect on the stability of the rotavirus at 30°C
30 (compare B and C, Table 7). This effect was greater at 37°C but still small compared to the standard (Formulation A). These data indicate that a wide concentration range (10-100%) of tissue culture medium is acceptable to attain improved stability.

Table 7
Potency loss (as log pfu/mL) of G1 rotavirus using formulations
with and without tissue culture medium.

	A	B	C
Loss after 1 week at 30°C	3.2	0.7	0.6
Loss after 1 week at 37°C	>6.5	0.6	1.0

5 To examine the effect of tissue culture medium at volume proportions of less than 10%, dialysis was employed to completely remove the tissue culture medium from the virus bulk. When a rotavirus liquid formulation was prepared from dialyzed virus bulk and thus contained 0% tissue culture media in the final formulation, these
10 preparations inactivated faster than preparations in which rotavirus bulk was simply diluted into a stabilizer without tissue culture media (resulting in 10% tissue culture medium being present in the final vaccine formulation). This suggests that dialysis may have removed essential stabilizing components that are present in WE tissue culture
15 medium. In the absence of an effective amount of tissue culture medium, divalent cations such as calcium can be added to the dialyzed vaccine formulation to improve stability (see Table 5). Dialysis at various processing scales can also be performed using diafiltration or ultrafiltration methods.

20 The stability of G1 reassortants was studied over a range of pH. Rotavirus G1 reassortant was formulated in 0.3 M sodium phosphate/0.1 M sodium citrate/50% sucrose stabilizer at different pH values. The viral titer indicates that under accelerated stability conditions, the stability of G1 reassortant is greater in the range from
25 about pH 4.0 to about pH 8.0, particularly between about pH 5.0 to about pH 7.0. By "about pH" we mean within approximately 0.3 units of the stated pH value.

Table 8
Potency log loss of G1 rotavirus after 1 month at 30 or 37°C in 0.3 M sodium phosphate/0.1 M sodium citrate/50% sucrose stabilizer at various pH values.

	1 month at 30°C	1 month at 37°C
pH 3	4.6	>6
pH 4	1.3	>6
pH 5	1.3	1.5
pH 6	1.3	1.4
pH 7	1.4	2.2
pH 8	1.6	>6

5

b. Lyophilized Formulation Stability Data

The G1 vaccine showed a 0.3 log loss after one year at 22°C in a lyophilized formulation of 1% sucrose/4% mannitol/10 mM sodium phosphate at pH 7 (Figure 8). Formulations containing 1% sucrose/4% mannitol/75 mM sodium phosphate at pH 7 showed no significant losses after one year at temperatures of 22°C or below. P1 vaccines showed lower stability than the corresponding G1 formulations. In 1% sucrose/4% mannitol/10 mM sodium phosphate at 4°C for one year, the P1 reassortant shows a 0.4 log loss in titer when compared with the vaccine stored at minus 70°C (Figure 9). A similar formulation with higher phosphate shows a loss in infectivity of less than 0.2 logs. The P1 vaccine in a phosphate, sucrose and hydrolyzed gelatin stabilizer shows no significant loss after one year at 4°C. These lyophilized formulations were prepared either by 10-fold dilution of rotavirus bulk into stabilizer (final concentration of 10% tissue culture medium) by dialysis of rotavirus bulk into stabilizer (complete removal of tissue culture medium).

20

WHAT IS CLAIMED:

1. A liquid rotavirus vaccine formulation comprising:
 - a) at least one strain of rotavirus about 1×10^5 to about 1000×10^5 pfu/mL;
 - 5 b) Sugar about 1 to about 70% (w/v);
 - c) Phosphate about 0.01 to about 2 M; and
 - d) at least one Carboxylate about 0.05 to about 2 M.
- 10 2. The formulation of Claim 1 wherein said at least one carboxylate is selected from the group consisting of succinate, citrate, fumarate, tartarate, maleate and lactate.
- 15 3. The formulation according to Claim 1 wherein said sugar is selected from the group consisting of sucrose, mannitol, lactose, sorbitol, dextrose, fucose, trehalose, polyaspartic acid, inositol hexaphosphate (phytic acid), sialic acid or N-acetylneuraminic acid-lactose.
- 20 4. The liquid vaccine formulation of Claim 1 further comprising:
 - e) at least one diluent selected from the group consisting of Tissue culture medium, saline and water To volume.
- 25 5. The formulation of Claim 1 wherein the concentration of sugar is between about 5 to about 70%; the concentration of phosphate is between about 0.05 to about 0.3 M; and said at least one carboxylic acid is citrate or succinate at a concentration between about 0.05 to about 0.7M.
- 30 6. The formulation according to Claim 1 wherein the pH is between about pH 5.0 to about pH 8.0.
- 35 7. The formulation according to Claim 1 wherein said phosphate is selected from the group consisting of monophosphates, polyphosphates and phosphorylated compounds.
8. The formulation according to Claim 7 wherein said phosphorylated compounds are phosphorylated sugars.

9. A lyophilized rotavirus vaccine formulation comprising:
- a) at least one strain of rotavirus about 1 to about 1000×10^5 pfu/mL);
 - b) at least one sugar about 1 to about 20% (w/v); and
 - c) Phosphate about 0.05 to about 2 M.
- 5
10. The formulation according to Claim 9 wherein said at least one sugar is selected from the group consisting of Sucrose, Mannitol and Lactose.
- 10
11. The formulation of Claim 9 comprising
- a) at least one sugar selected from the group consisting of Sucrose and lactose about 1% (w/v);
 - b) Mannitol about 4% (w/v); and
 - c) Phosphate about 0.010 to about 0.075 M.
- 15
12. The formulation according to Claim 9 wherein the pH is between about pH 5.0 to about pH 7.0.
- 20
13. A method of preparing stabilized rotavirus vaccine formulations, comprising:
- (a) cultivating a rotavirus and mixing the rotavirus with a concentrated stabilizing solution to form a concentrated mixture; and, optionally,
 - (b) dialyzing the virus bulk or concentrated mixture
- 25 to form a stabilized rotavirus vaccine solution.
- 30
14. A method of administering an oral rotavirus vaccine formulation to an individual comprising treatment of the individual with a compound having sufficient buffering capacity to neutralize stomach acid, wherein the treatment is before, coincident with or subsequent to the administration of the vaccine.

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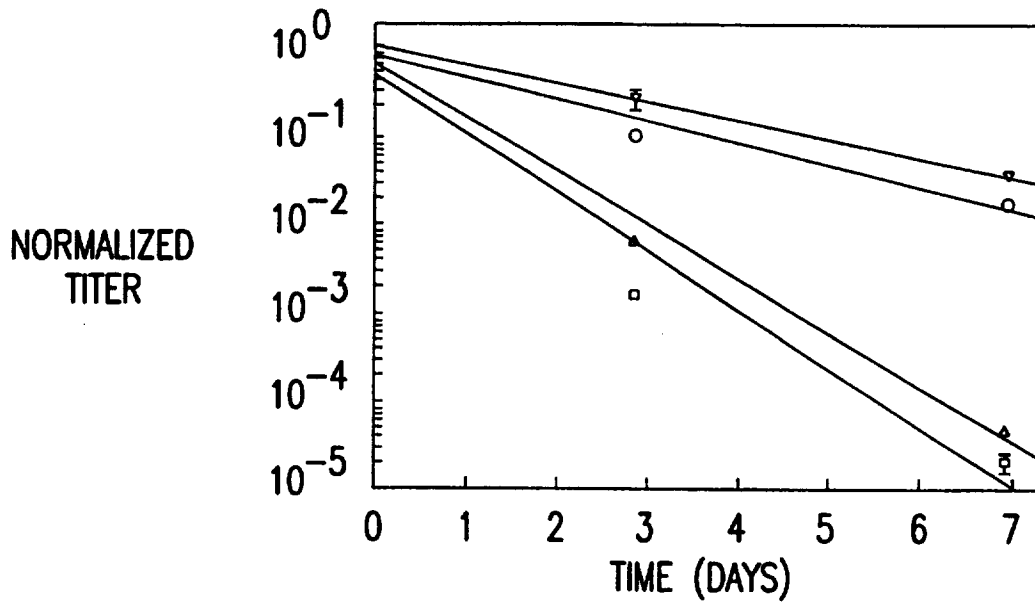


FIG. 1A

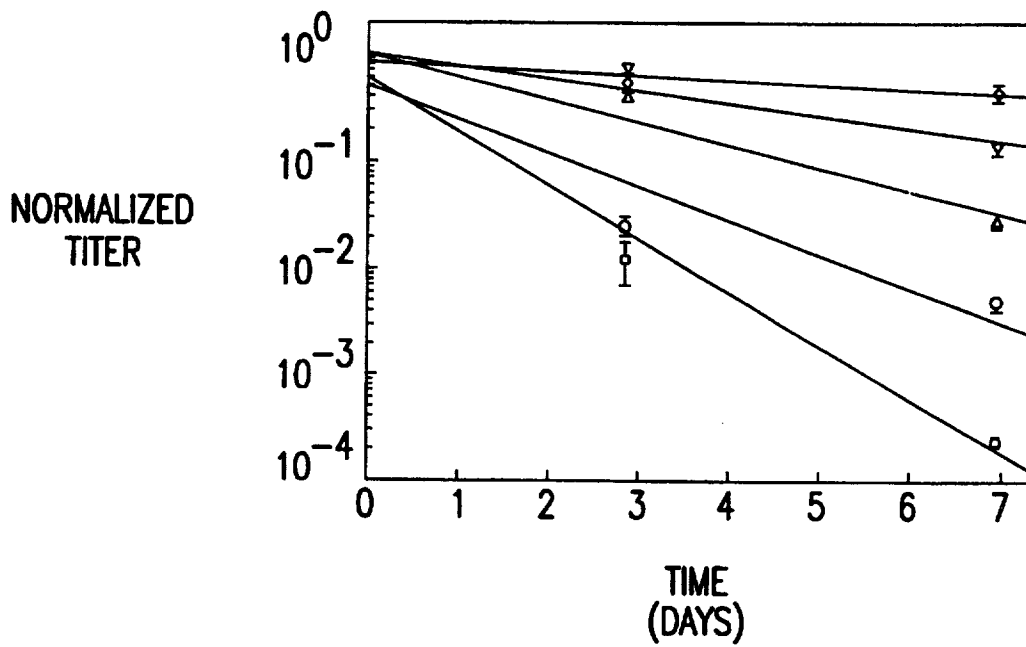


FIG. 1B

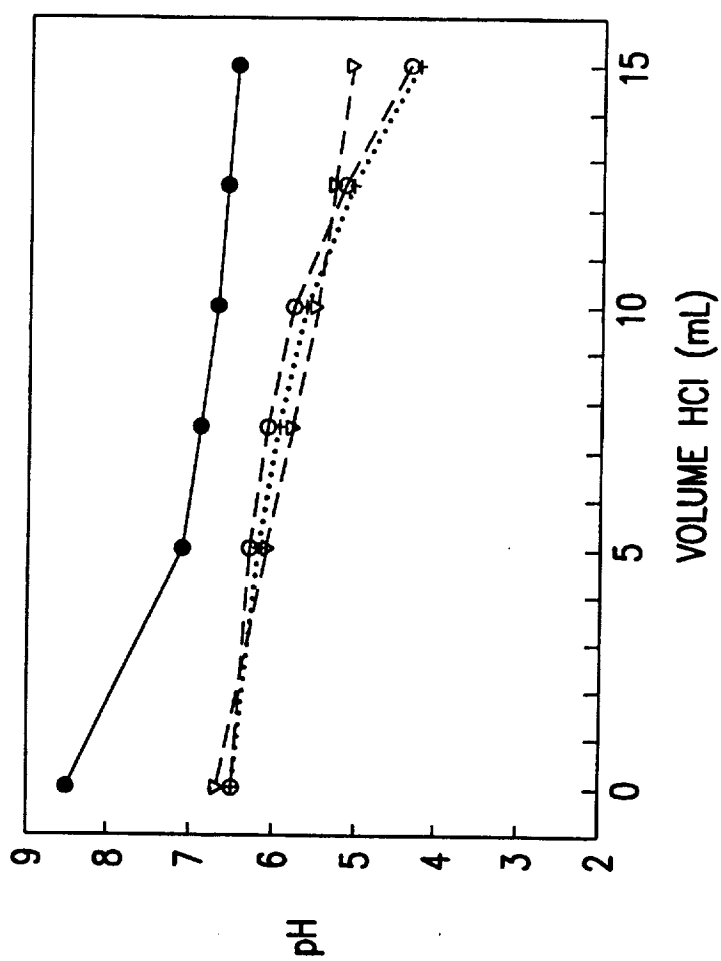


FIG.2

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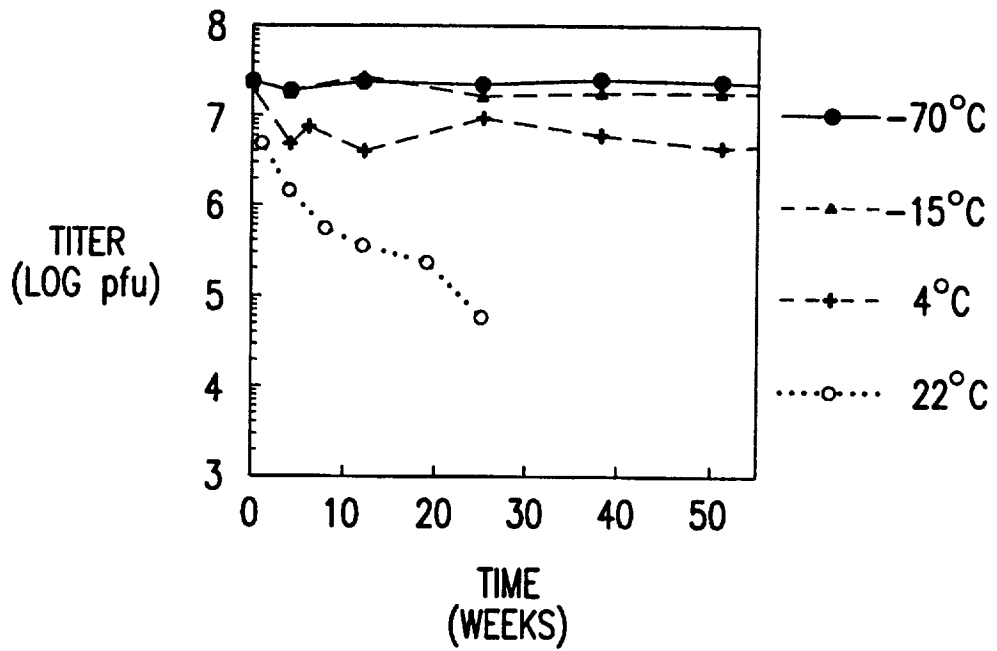


FIG.3A

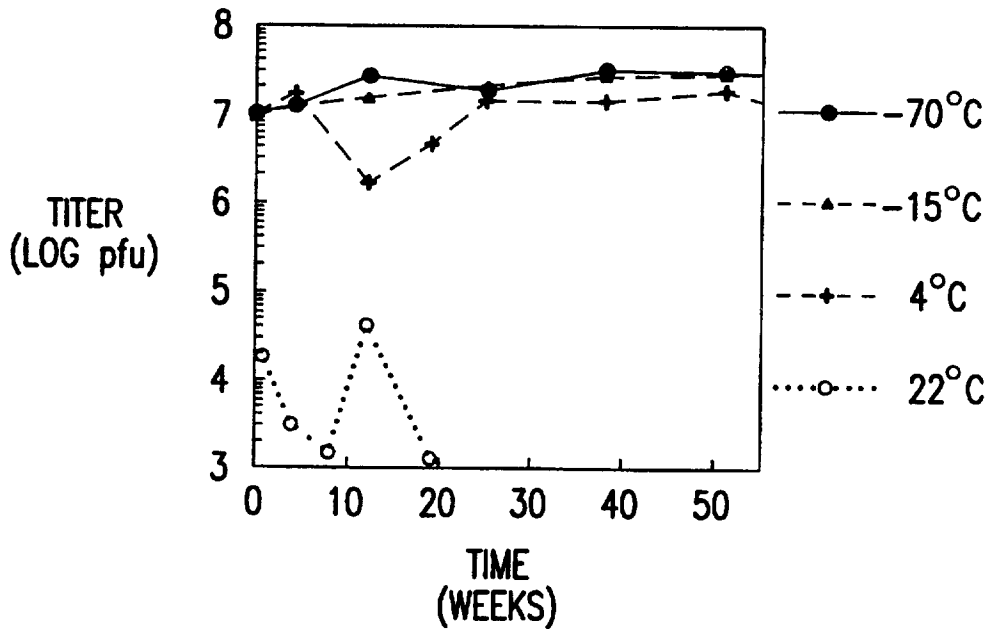


FIG.3B

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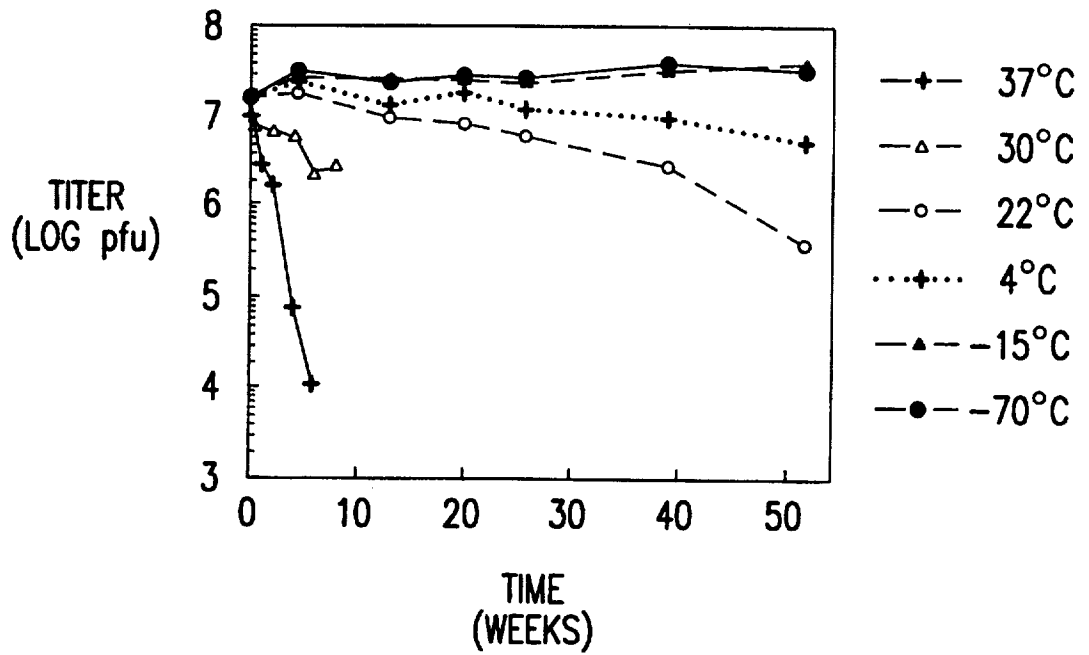


FIG.4A

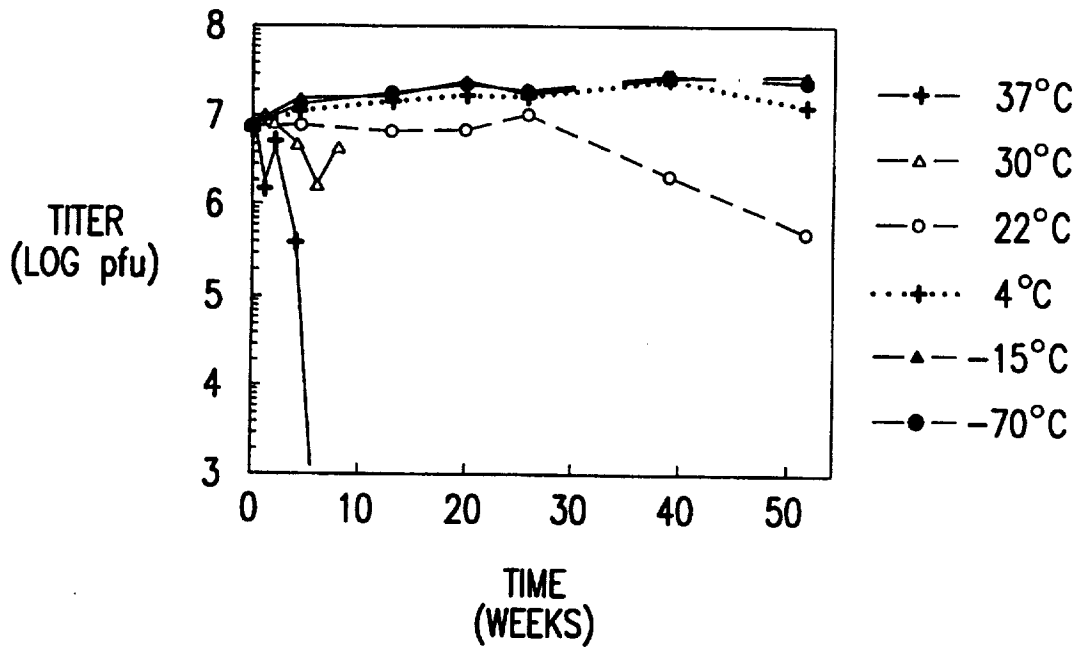


FIG.4B

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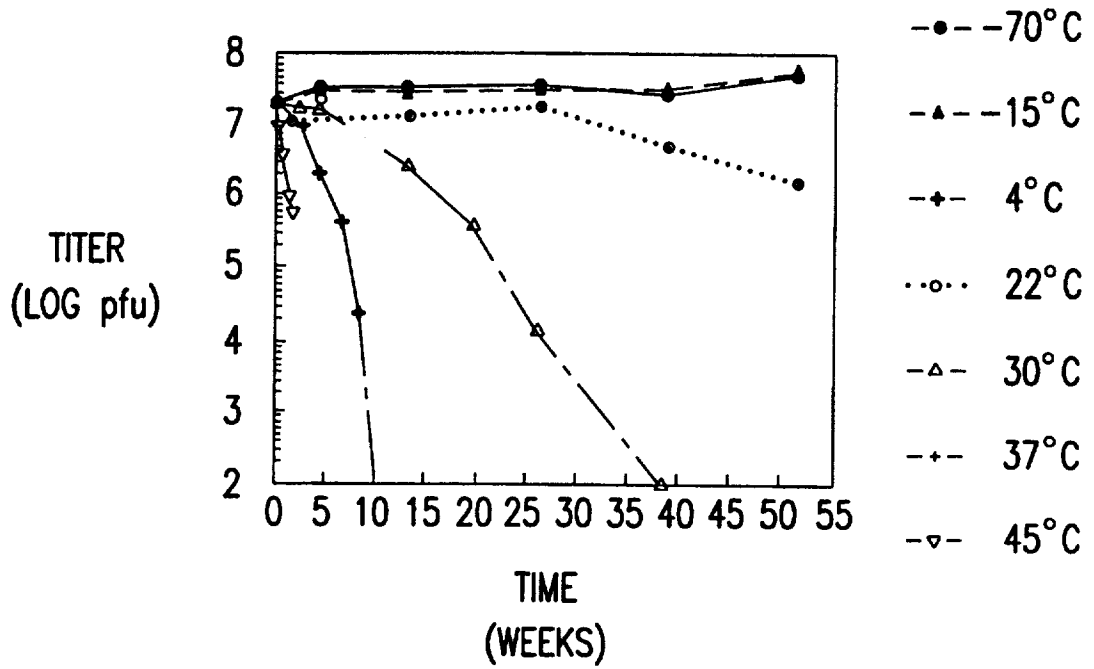


FIG.5A

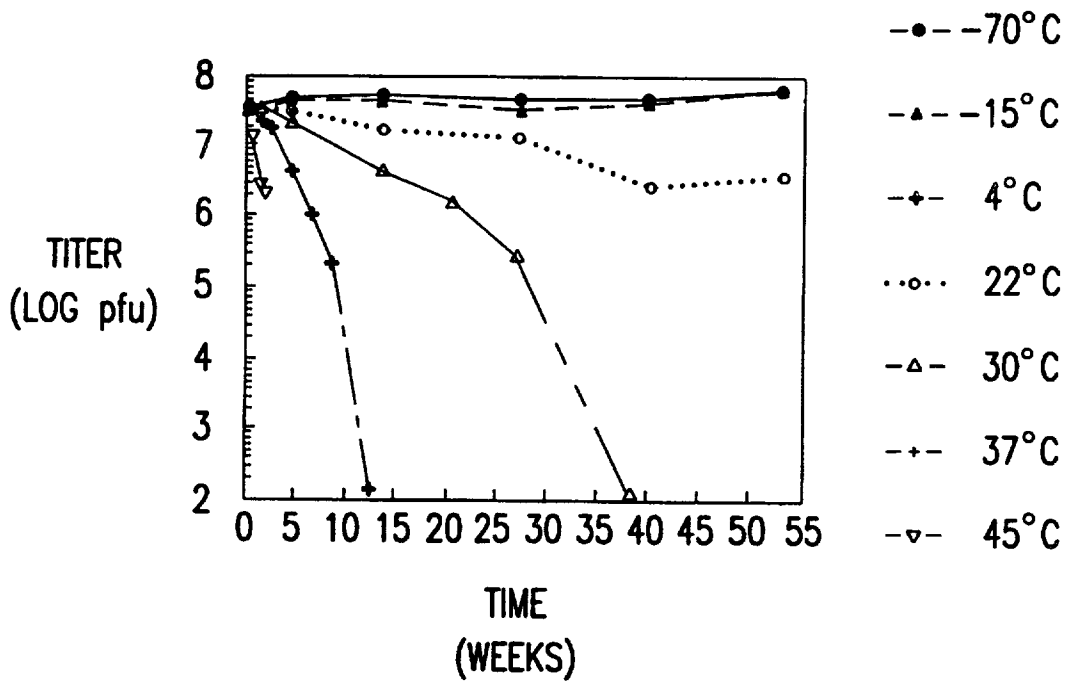


FIG.5B

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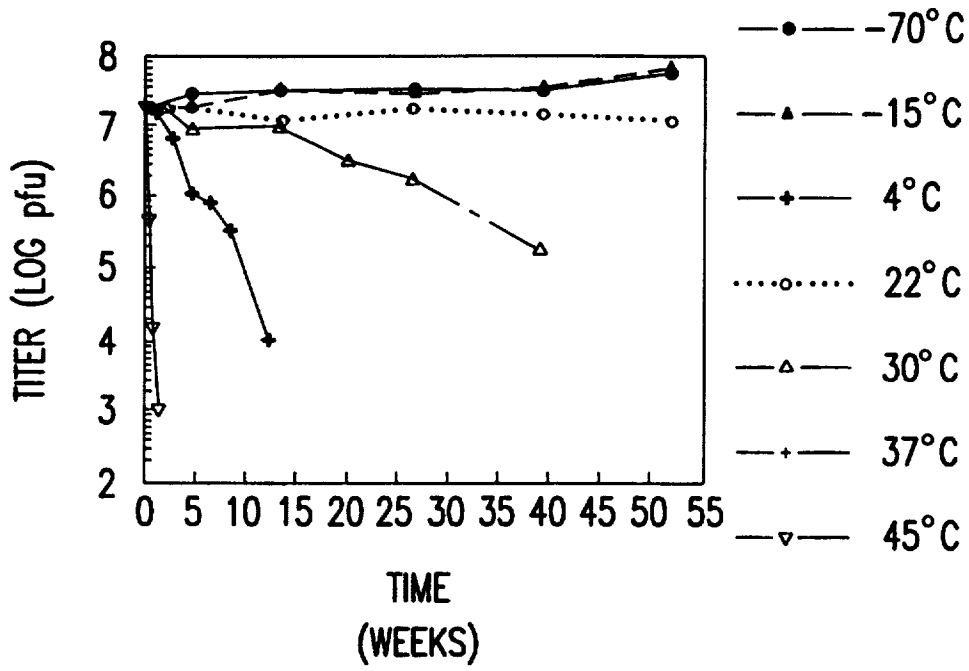


FIG. 5C

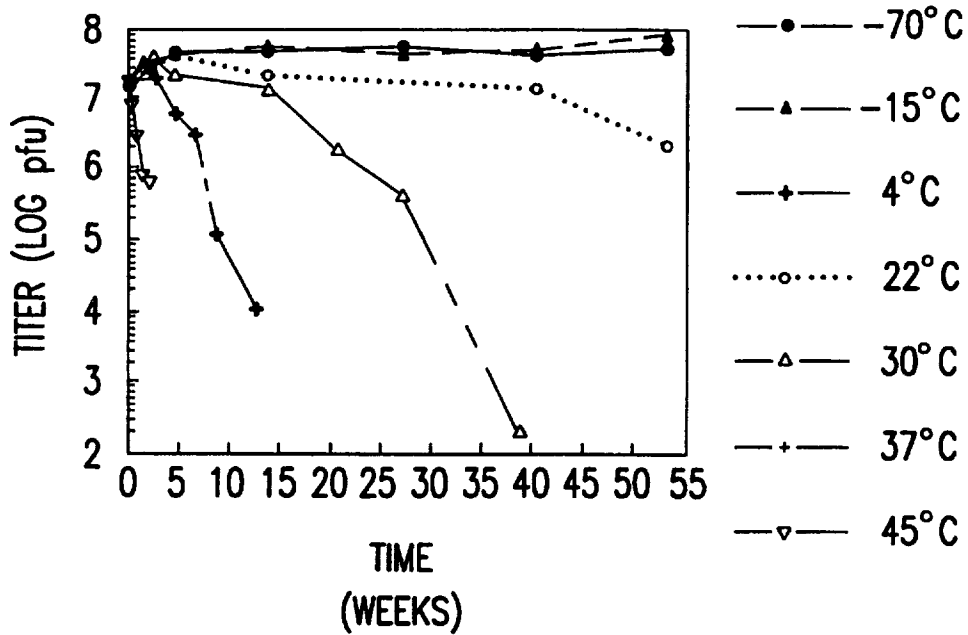


FIG. 5D

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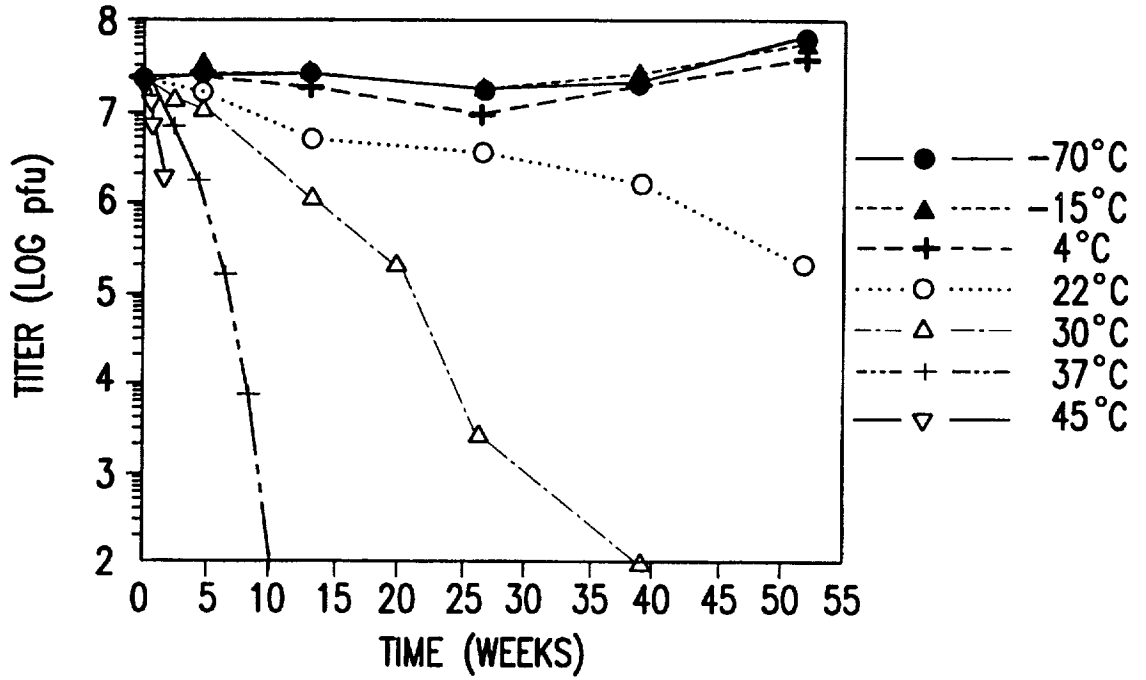


FIG.6A

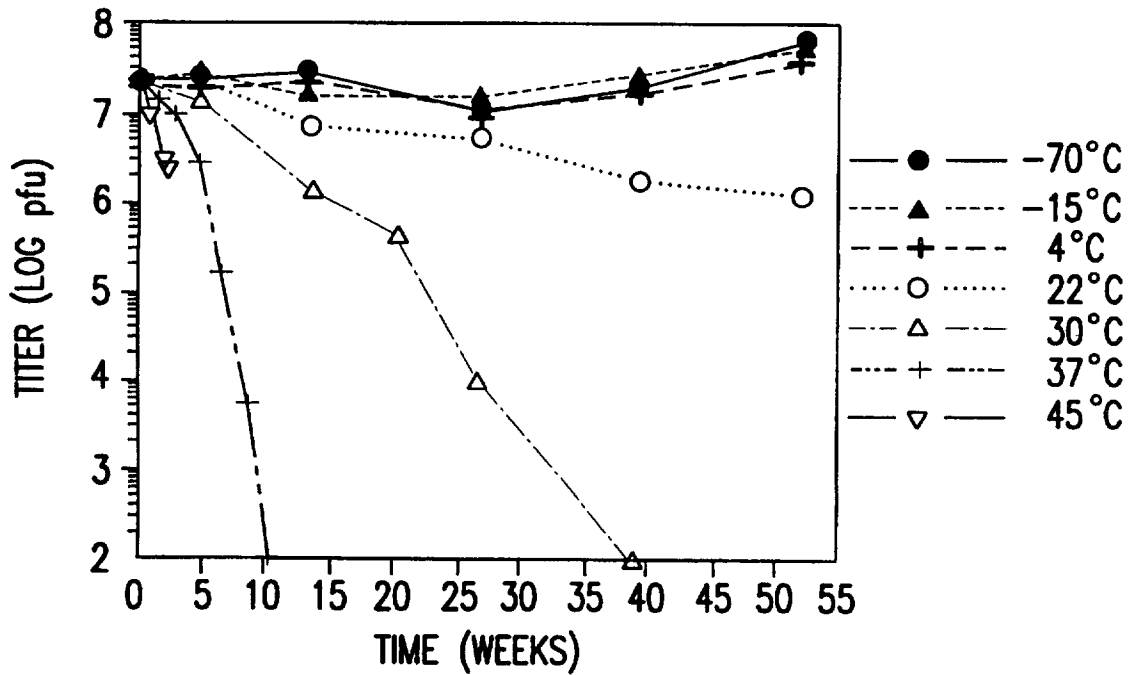


FIG.6B

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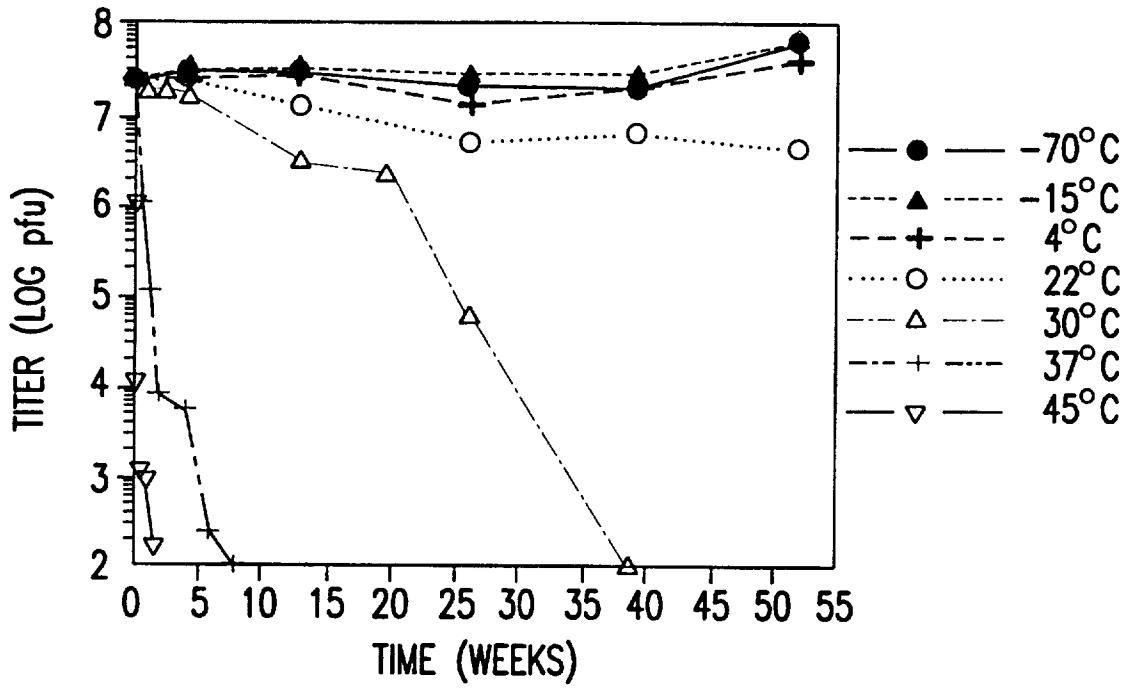


FIG.6C

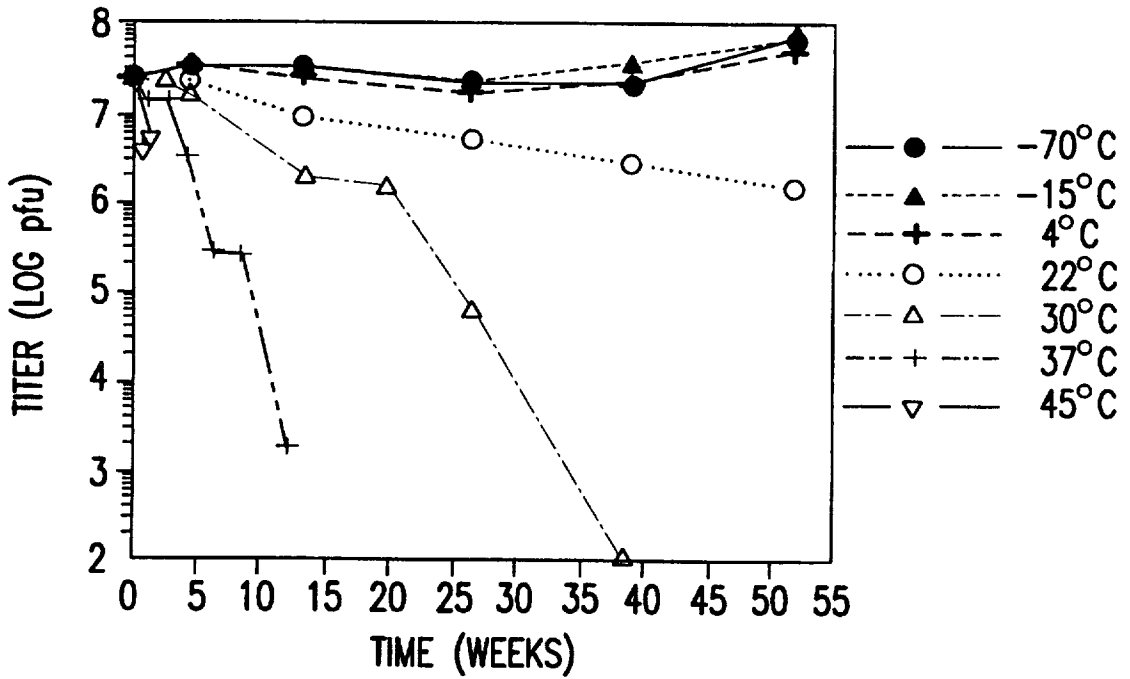


FIG.6D

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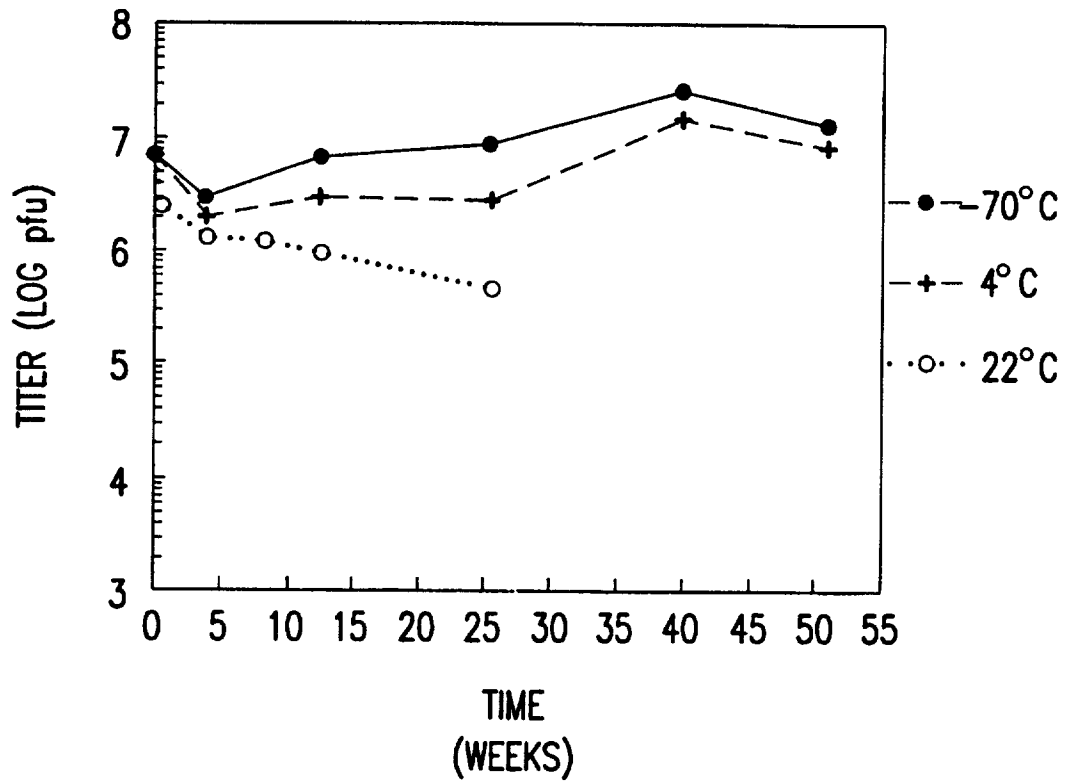


FIG.7A

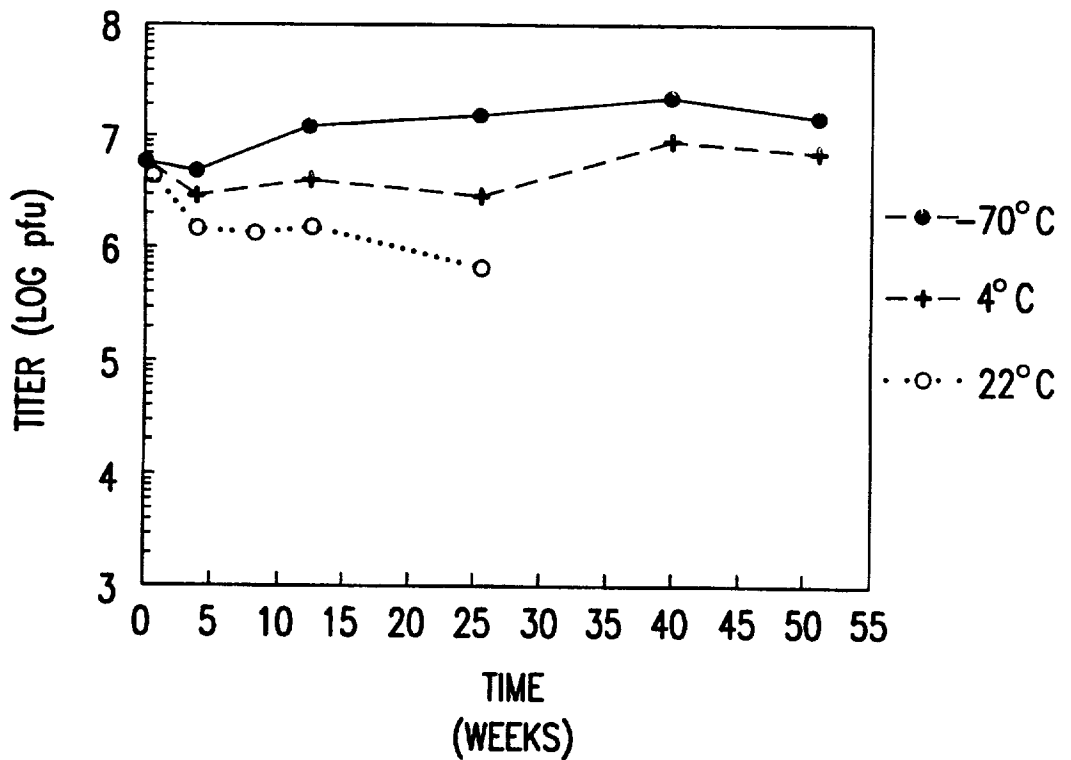


FIG.7B

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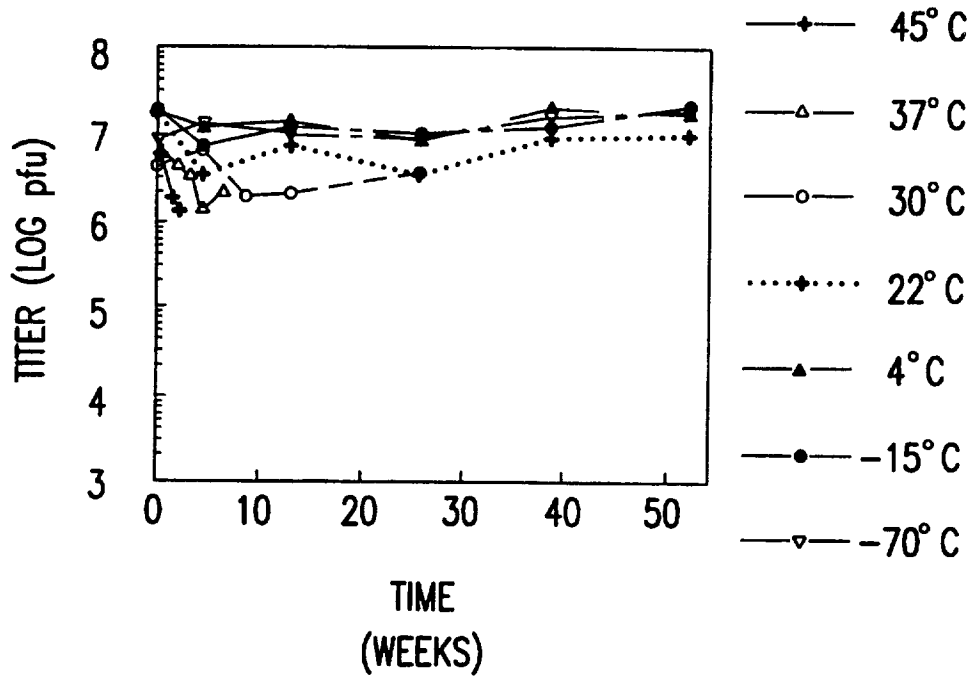


FIG. 8A

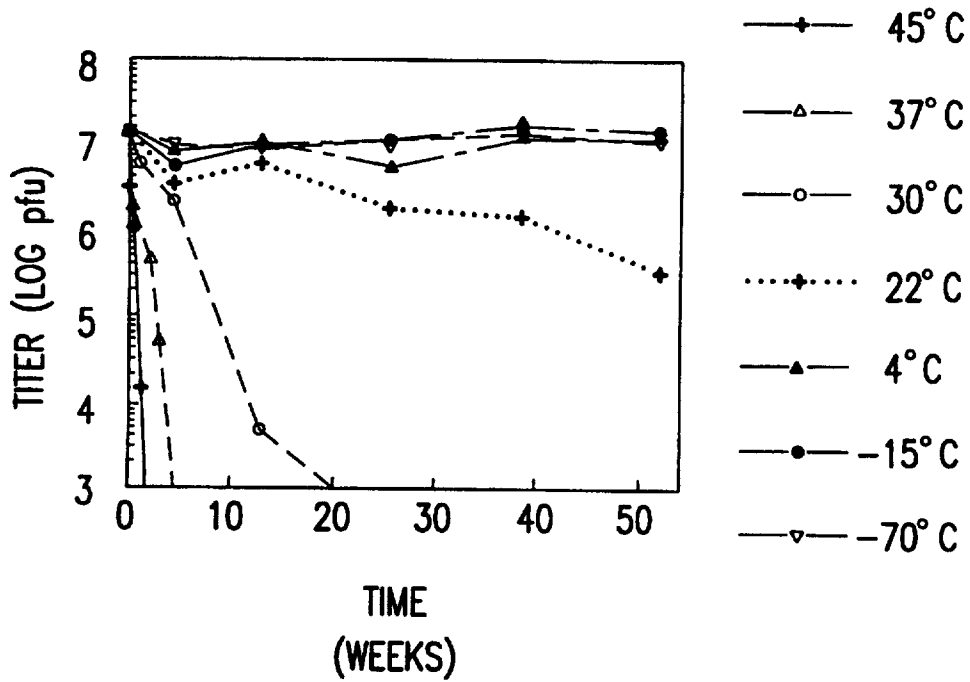


FIG. 8B

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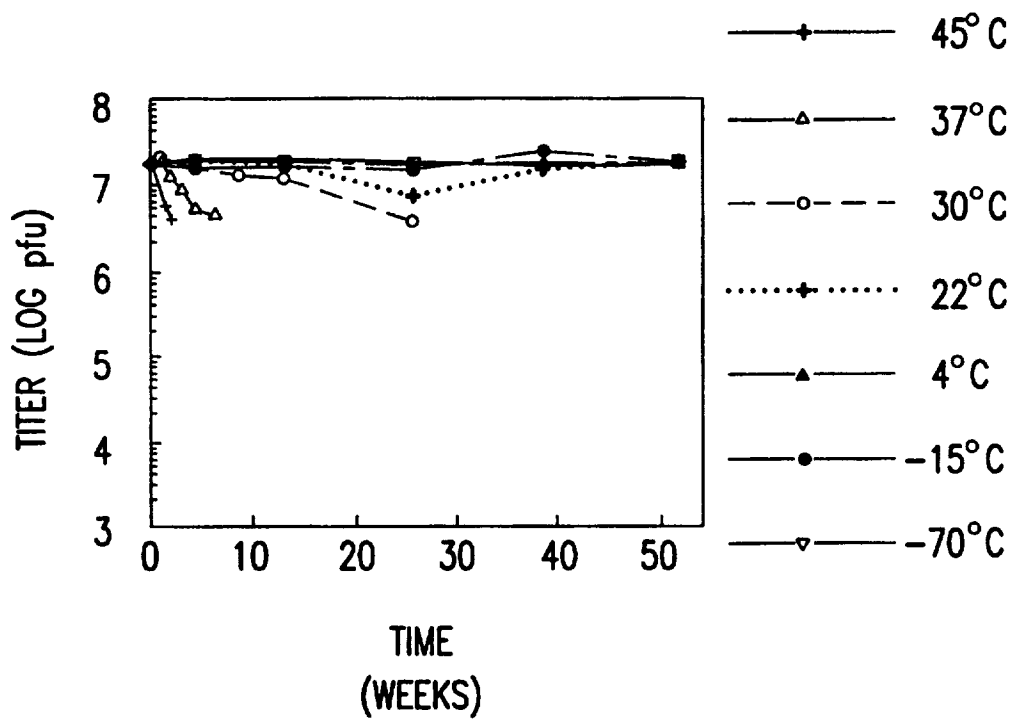


FIG.8C

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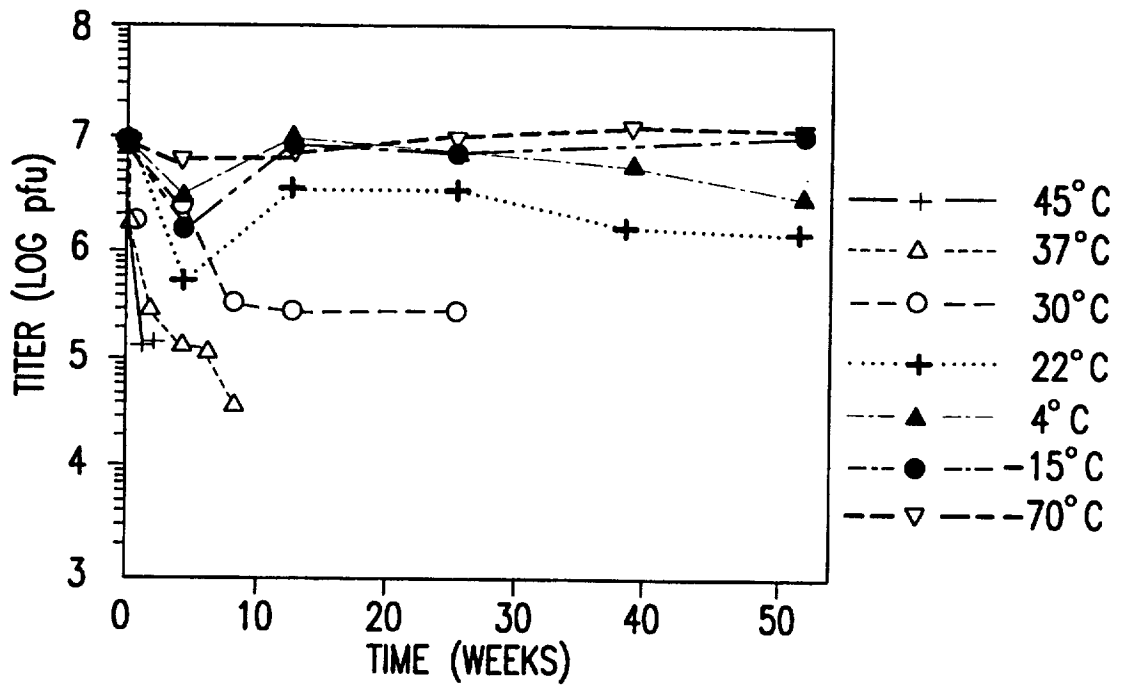


FIG.9A

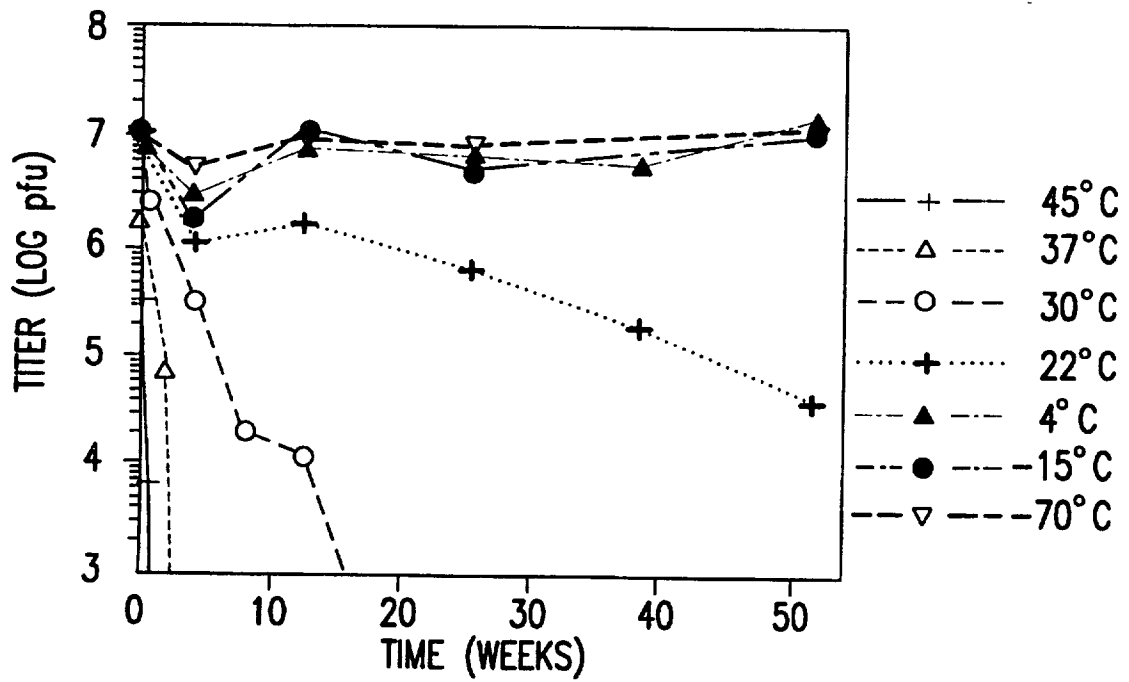


FIG.9B

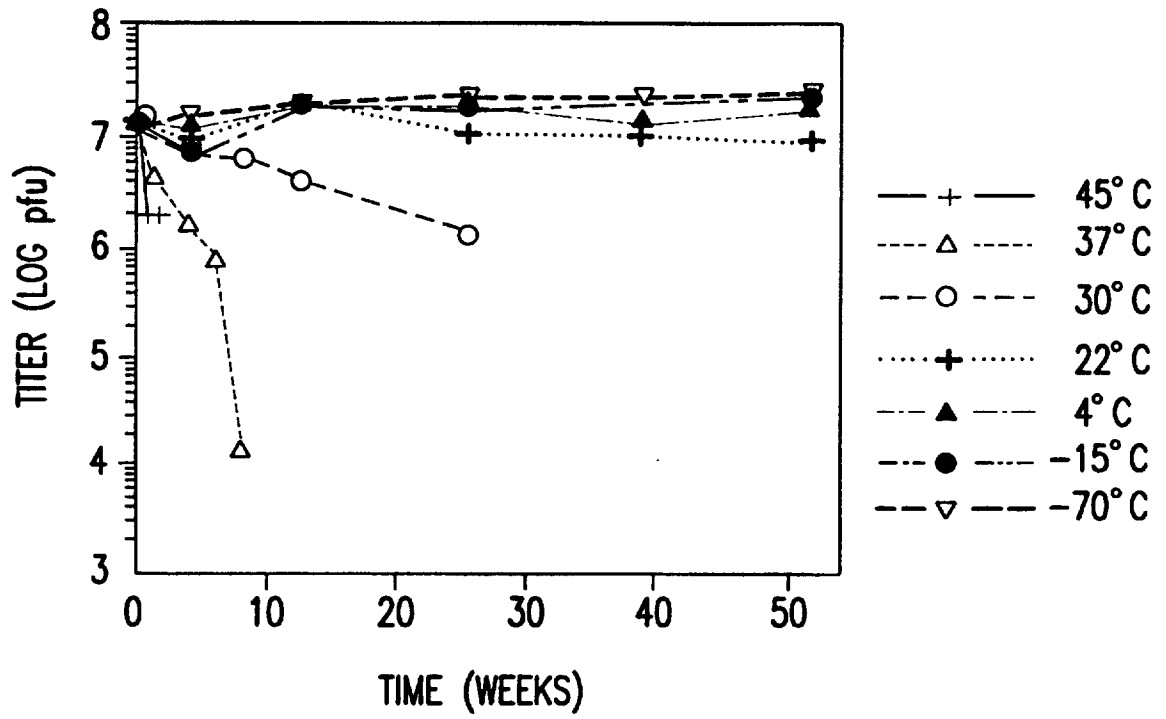


FIG.9C

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/17274

A. CLASSIFICATION OF SUBJECT MATTER IPC 6 A61K39/15				
According to International Patent Classification (IPC) or to both national classification and IPC				
B. FIELDS SEARCHED				
Minimum documentation searched (classification system followed by classification symbols) IPC 6 A61K				
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched				
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)				
C. DOCUMENTS CONSIDERED TO BE RELEVANT				
Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.		
P,X	US 5 626 851 A (H. FRED CLARK ET AL) 6 May 1997 cited in the application see column 7, line 66 - column 8, line 42 see claims 1-39 ---	1-5,12		
A	WO 96 01651 A (THE GOVERNMENT OF THE UNITED STATES OF AMERICA...) 25 January 1996 see page 11, line 9 - line 34 see claims 1-6,19-21 ---	1-13		
A	EP 0 192 404 A (THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY) 27 August 1986 see claims 10-17 see page 5, line 28 - page 6, line 4 & US 4 636 385 A cited in the application ---	1-13		
-/--				
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.				
<input checked="" type="checkbox"/> Patent family members are listed in annex.				
° Special categories of cited documents :				
<table style="width: 100%; border: none;"> <tr> <td style="width: 50%; vertical-align: top; padding: 5px;"> *A* document defining the general state of the art which is not considered to be of particular relevance *E* earlier document but published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed </td> <td style="width: 50%; vertical-align: top; padding: 5px;"> *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. *&* document member of the same patent family </td> </tr> </table>			*A* document defining the general state of the art which is not considered to be of particular relevance *E* earlier document but published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. *&* document member of the same patent family
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9 February 1998	03.03.98			
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INTERNATIONAL SEARCH REPORT

Intern. Application No
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