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COMPLETE SPECIFICATION

Improvements in and relating to Preservation of Cells

We, UNION CARBIDE CORPORATION, of 270, Park Avenue, New York, State of New York, United States of America, a Corporation organised under the laws of the State of New York, United States of America, (Assignee of SHELDON WALTER MOLINE and ARTHUR WILSON ROWE), do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to a process for preserving cells. More particularly, the invention is directed to a process for preserving at low temperatures cells derived from animal tissue.

Viable cells obtained from tissues of various animals have many important uses. For example, cells obtained from animal organs, such as kidney cells, provide growth media for virus particularly in the production of vaccines. Bone marrow, another form of tissue, is being used increasingly in medical practice in treatment of certain types of anemia, cancer therapy, and in treatment for exposure to high levels of radiation.

It is an object of this invention to provide a process for the low temperature preservation of viable cells obtained from animals.

Another object of this invention is to provide a method for preserving viable cells over long periods of time.

A further object of this invention is to provide a means for preserving tissue cells in a condition suitable for supporting virus growth in vaccine production.

These and other objects and advantages of this invention will be apparent from the following description and appended claims.

The term "tissue," as used herein, means an aggregate of cells, with their intercellular substance, forming one of the structural materials of an animal. This definition includes tissue in the form of body organs and

bone marrow, but excludes body fluids, blood, and cells originally present in the animal as individual cells, such as erythrocytes and leucocytes.

Broadly stated, the process of this invention comprises the steps of (1) contacting animal tissue cells with a protective additive to reduce cell damage during freezing and thawing; (2) cooling the cell-additive combination to freezing point and then making the liquid to solid phase change in less than 10 minutes, latent heat of fusion being removed; (3) further cooling the solidified cells to -50°C . at a rate of not more than 3°C . per minute, and (4) further cooling the frozen cells and maintaining the frozen cells at a temperature below -75°C .

The process of this invention is generally applicable to the preservation of tissue cells from any animal, preferably mammals. For example, bone marrow and tissue of kidneys, liver, heart, spleen and lungs, obtained from birds, reptiles, fish, rabbits, monkeys, cats, dogs, rodents, horses and human beings can be preserved by the process of this invention.

Cells for use in the process of this invention are obtained from donor animals by conventional procedures. Several procedures for obtaining various types of cells and cell aggregates are described in detail in the discussion which follows.

The process of this invention is applicable to tissue in the form of cell aggregates of relatively small size, as for example, bone marrow or finely minced tissue; as well as to the preservation of tissue in the form of large cell aggregates including whole organs such as kidneys and lungs. The process of this invention is also applicable to cells originally present in the animal as part of a tissue cell aggregate but which have been broken down into individual cells prior to freezing.

The cells to be preserved are contacted with a protective additive which reduces damage to the cells during the freezing and



particularly

[Price 4s. 6d.]

Com	
DL	
D	
DL-serum	
5	DL-threonine
	DL-leucine
	DL-isoleucine
	DL-valine
	DL-glutamic acid monohydrate
10	DL-aspartic acid
	DL-alpha-alanine
	L-proline
	L-hydroxyproline
	Glycine (aminoacetic acid)
15	L-glutamine
	Sodium Acetate Trihydrate
	L-cystine
	L-tyrosine
	L-cysteine hydrochloride
20	Adenine
	Guanine
	Xanthine
	Hypoxanthine
	Uracil
25	Thymine (5-methyl uracil)
	Disodium Alpha Tocopherol Phosphate
	Thiamine
	Pyridoxine Hydrochloride
30	Riboflavin
	Pyridoxal Hydrochloride
	Niacin
	Niacinamide
35	Calcium Pantothenate
	i-inositol
	Ascorbic Acid
	Folic Acid
	p-Aminobenzoic Acid
	Ferric Nitrate
40	Biotin
	Menadione
	Glutathione
	Vitamin A
	Calciferol
45	Cholesterol
	Tween 80*
	Adenosinetriphosphate (sodium salt)
	Adenylic Acid
50	Desoxyribose
	D-ribose
	Choline Chloride

*Trademark of Atlas Powder Co.

55	(C)	10% egg yolk
		20% serum
		10% dimethylsulfoxide (CH_3SCH_3)
		60% Eagle's MEM

(D)

10% egg yolk
20% serum
10% dimethylsulfoxide
60% TC-199

(E)

20% serum
10% dimethylsulfoxide
70% Eagle's MEM

(F)

10% Centrex F
20% serum
10% dimethylsulfoxide
60% Eagle's MEM

"Centrex F" is a commercially available nutrient medium comprising a 1% by weight dispersion in water of a crude soya bean phospholipid fraction. The phospholipid fraction consists essentially of a 1:1:1 mixture of lecithin, cephalin, and phosphatidylcholine.

(G)

2.5 wt.% glucose
0.6 wt.% methylcellulose (15 centipoise viscosity)
20% serum
16.9% water
10% dimethylsulfoxide
50% Eagle's MEM

The methylcellulose improves the efficiency of the perfusion step by increasing the viscosity of the perfusion medium and also enhances the integrity of cell membrane during trypsinization. The serum in the above media (A)-(G) can be autologous serum, homologous serum or heterologous serum.

The cells to be frozen are cooled to the freezing point and then the liquid to solid phase change made in less than 10 minutes, removing latent heat of fusion. This cooling is carried out by conventional methods, for example, by immersion in a liquid refrigerant such as liquid nitrogen or by passing cold gas over the material to be frozen. Apparatus is available commercially for freezing biological substances by these methods. The terms "solidification" and "liquid-solid phase change" as used herein, apply to both (a) liquid in which tissue cells or cell aggregates are suspended and (b) liquid present within the solid walls of individual cells or within the solid matrix of tissues and organs.

It is important for the solidified tissue cells, after the liquid-solid phase change is completed, to be cooled to a temperature of -50°C . at a rate not in excess of 3°C . per minute. It has been found that cooling

at faster peratur
 5 from the solidification tem-
 -50° C results in reduced
 the cells. The temperature of
 presents the approximate tem-
 with all freezable water in the
 converted to solid. Certain
 cal systems, for example,
 proteins or other hydrophilic
 not freezable. The exact tem-
 10 perature which all freezable water solidifies
 varies somewhat with the particular system
 involved, but in general all freezable water
 will have solidified at a temperature of -50°
 C.
 15 Once the temperature of -50° C. has
 been reached the frozen cells can be further
 cooled at any desired rate to a temperature
 below -75° C. Any refrigerant capable of
 producing temperatures below -75° C. can
 20 be employed, for example, solid carbon
 dioxide, solid carbon dioxide-solvent mix-
 tures, helium, neon, argon and nitrogen. A
 storage temperature of -75° C. is adequate
 for short storage periods. For longer storage,
 25 temperatures below -130° C. are pre-
 ferred. At liquid nitrogen temperatures
 (-170° C. down to -196° C.)

via ind fri larg toxic tures free well as cells at teni
 almost re in 30
 and non-mer, low tempera-
 paratus for use in
 controlled rates, as
 storing frozen tissue 35
 from -75° C. to -196°
 C. are commercially available.

When the tissue cells preserved by the
 process of this invention are desired for
 further use, the frozen cells are thawed rapidly
 by conventional methods, preferably by
 means of a warm water bath, usually main-
 tained at 37° C. Where a frozen suspension
 of single cells or cell aggregates (such as
 bone marrow or minced tissue) is to be
 40 thawed, the container holding the frozen cells
 can be immersed in a water bath and the
 contents agitated during the thawing process.
 Where a frozen whole organ is to be thawed,
 it is preferable to immerse the frozen organ
 in an isotonic warming fluid rather than
 45 directly in water. A suitable isotonic solution
 is "GKN" which has the following com-
 positions:

55
 Glucose—1.0 g.
 KCl —4.0 g.
 NaCl —8.0 g. } —per 1000 ml. of aqueous solution

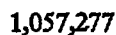
60 The warming fluid is maintained at a tem-
 perature of 37° C. and may be agitated to
 enhance heat transfer to the immersed organ
 if desired. Thawing is preferably carried out
 as rapidly as possible, but the thawing fluid
 should not exceed the normal body tempera-
 ture of the organ.

65 The thawed cells can then undergo further
 processing depending upon the desired use.
 For example, when tissue cells are needed to
 provide a growth medium for a virus as in
 vaccine production, the thawed minced organ
 (or a mince prepared from a thawed whole
 organ) is subjected to a standard trypsiniza-
 70 tion procedure which serves to break down the
 aggregates into individual cells, to disperse
 the cells, and to dilute out the protective
 additive. The individual cells are then
 cultured by standard methods to provide a
 75 growth medium for the virus.

80 Where bone marrow preserved by the pro-
 cess of this invention is to be used for trans-
 fusion into human beings and intracellular
 protective additives (such as glycerol or di-
 methylsulfoxide) are used, special post-thaw
 processing is required. The special processing
 involves partial removal of the protective addi-
 tive by slow dilution techniques. It is essential
 85 that the additive concentration be reduced
 before transfusing the cells into a human
 being, since direct infusion leads to immediate
 osmotic shock to the cells. A suitable diluting

90 procedure is to add one-half volume of physio-
 logical saline solution (0.85 wt.% NaCl) to
 one volume of the thawed bone marrow sus-
 pension. Five to ten minutes later, one volume
 of saline is added to one volume of the pre-
 viously diluted cell suspension. This procedure
 is repeated after an additional five to ten
 95 minutes, resulting in approximately a six-
 fold dilution of the original suspension. The
 dilute bone marrow suspension is then con-
 centrated by centrifuging and discarding the
 supernatant liquid until the desired volume
 is attained. It is desirable to reduce the con-
 100 centration of protective additive to below
 2.5% prior to transfusion. Glucose solutions
 can also be used as the diluting medium.

105 In a preferred embodiment of the inven-
 tion, the tissue cells to be preserved are cooled
 through the phase change as rapidly as
 possible, and in any event in less than 10
 minutes. Preferably the cell aggregates are
 cooled through the phase change in 2 minutes
 or less. Cooling through the phase change can
 be carried out most rapidly and efficiently on
 organs or on small tissue cell suspensions.
 However, relatively large tissue cell suspen-
 110 sions can be cooled through the phase change
 in less than 10 minutes by using a container
 having large surface-to-volume ratio and a
 low temperature refrigerant such as liquid
 nitrogen. The increased viability of the frozen
 cells which results from rapid cooling through



It may be necessary to combine the marrow

k. The dorsal aorta

aluminum

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114

12

125

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cell aggregates, such as, or equivalent, with a suitable anticoagulant, citrate-dextrose (ACD), heparin, ethylenediamine tetraacetic acid (EDTA). The concentrations used are those ordinarily used for standard anticoagulation of blood. The anticoagulant is most necessary for preventing clotting of contaminating peripheral blood in the marrow sample rather than the marrow cells themselves. Where the marrow is obtained by sacrifice of the donor animal, it is often not necessary to use an anticoagulant because the peripheral blood contamination can be kept to a minimum when the marrow-containing bone is excised or the animal is exsanguinated prior to marrow collection.

The bone marrow, with or without anticoagulant, is then added to a suspending medium, and the resulting suspension is combined with a protective additive solution. The suspending medium employed is a standard balanced salt solution, for example, Tyrode's or Hank's media which have the following compositions expressed in grams per liter of solution:

	Tyrode's	Hank's
NaCl	8.00	8.00
KCl	0.20	0.40
CaCl ₂	0.20	0.14
MgCl ₂ · 6H ₂ O	0.10	—
MgSO ₄ · 7H ₂ O	—	0.20
Na ₂ HPO ₄ · 12H ₂ O	—	0.12
NaH ₂ PO ₄ · H ₂ O	0.05	—
KH ₂ PO ₄	—	0.06
NaHCO ₃	1.00	0.35

NaCl	8.0 g.
KCl	0.2 g.
MgCl ₂ · 6H ₂ O	0.1 g.
Na ₂ HPO ₄	1.15 g.
KM ₂ PO ₄	0.2 g.
CaCl ₂	0.1 g.
Trypsin (1—300 grade)	1.0 g.

per 1000 ml of aqueous solution

Trypsin is a proteolytic enzyme that catalyzes the hydrolysis of peptide linkages in proteins. Trypsinization is a standard biochemical procedure used to break down tissues into their constituent cells.

For 5 grams of minced tissue, a total of 100 ml. of buffered trypsin solution is added, five 10 ml. aliquots being added at three-minute intervals, followed by the addition of the final 50 ml. Once all the trypsin solution has been added, the suspension is stirred for 10 minutes, the particles allowed to settle and the supernatant fluid is removed by decantation and discarded. This procedure is preferred since freshly trypsinized cells may liberate a cytotoxin, (i.e. a material which is

toxic to the cells) which should be removed or it may destroy the suspended cells.

Following the removal of the cytotoxin, 100 ml. of the trypsin solution are added to the tissue fragments and the suspension is stirred for 30 minutes at room temperature. The tissue fragments are allowed to settle and the supernatant fluid containing dispersed cells is decanted into a centrifuge bottle. The cells are then collected by centrifugation and resuspended in GKN solution containing 10% serum and maintained at 0° C. This trypsinization step is repeated one or more times in order to completely break down the tissue fragments into their component cells. Use of GKN containing serum at this stage is based

GK
Ph

Hanks'

90

The media described above are used alone or in combination with other media of the class described. Varying amounts of serum are commonly added to the suspending medium for nutrient purposes and to stabilize the cells. A typical suspending medium comprises Hank's balanced salt solution containing 10 to 20 volume per cent pooled homologous serum i.e. a mixture of serums of several animals belonging to the same species. A protective additive of the class described above is then added and the resulting suspension is frozen by the methods of this invention.

Typical procedures for isolating individual cells from thawed cell aggregates and for preparation of cell cultures are as follows:

Where minced tissue has been frozen in suspension, the thawed suspension can be used directly. Where a whole organ has been frozen, the thawed organ is minced and suspended in one of the suspending media described hereinabove.

In order to carry out the cell separation procedure, the suspension of minced tissue is placed in a trypsinizing flask. A solution of trypsin in a buffered salt solution containing 0.12% methylcellulose (15 centipoise grade) is added slowly with stirring. The trypsin concentration depends on the type of tissue and is usually in the range of 0.1 to 0.27 weight per cent. The composition of a typical buffered trypsin solution is as follows:

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agent
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To prepare the
resuspended in a
conventional media

10 that has proved satisfactory, a mixture of
40% serum and 60% Eagle's MEM contain-
ing 10 ml. of 200 millimolar glutamine solu-
tion per liter of mixture. An aliquot of the
suspension of cells in growth medium is re-
15 moved for counting in order to provide a
control sample. Finally, aliquots containing
 1×10^4 to 2×10^6 cells/ml. are placed in
growth vessels and incubated at 37°C . in
an atmosphere composed of 5% carbon
20 dioxide and 95% air.

The process of this invention can be used
to preserve individual cells which have been
obtained from tissue cell aggregates by the
above described trypsinization procedure (or
25 other comparable methods for obtaining
single cells from cell aggregates) or
the cell aggregates can be pre-
served by the process of this invention and
later broken down into individual cells if so
30 desired.

A convenient method for comparing the
viability of frozen and thawed cells with un-
frozen controls is to compare the rate of
growth of cells in a culture medium over a
35 period of time of up to 2 weeks. When the
culture medium is inoculated with cells from
an unfrozen control with a concentration of
 5×10^5 cells per milliliter and the growth
cells incubated at 37°C . in an atmosphere
40 comprising 5% carbon dioxide and 95%
air, the cell concentration decreases slightly
over the first few days and then increases
toward a concentration of 1×10^6 to 2×10^6
cells/ml. The unfrozen control cells reach
45 a concentration of 1×10^6 to 2×10^6 cells/ml.
after 7 to 9 days of growth. The viability of
the frozen and thawed cells can then be
evaluated by the number of days of growth
required to reach the cell concentration of
50 1×10^6 to 2×10^6 cells/ml. When the frozen
and thawed cells reach this concentration
within 3 days longer growth than the un-
frozen controls, the viability of the cells can
be considered satisfactory. In a similar
55 manner, the relative viability of two or more
samples of frozen and thawed cells can be
compared by the length of time necessary for
the cultured cells to reach a concentration of
 1×10^6 to 2×10^6 cells/ml.

60 A second method for evaluating viability
of cells is the glycine incorporation technique.
This method is based on the protein syn-
thesizing capacity of the preserved cells. The
technique used measures the uptake and in-
65 corporation of an amino acid labeled with

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then

a radioactive isotope, that measures the
ability of the cell to admit no acid
and then to incorporate it in protein
fraction of the cell. The acid
labeled glycine (amino-acid)
test shows whether or not
of actively transporting gly
terior and also whether t
present inside the cell, can
1 for
protein synthesis. In order to as glycine
75 incorporation, cells which have been subjected
to the freeze-thaw procedure are incubated
with glycine- 2-C^{14} at 37°C . and aliquots
are removed at various time intervals. The
acid-insoluble protein fraction of the cells is
80 precipitated with cold, 10% trichloroacetic
acid. The precipitate is washed with cold
5% trichloroacetic acid, then washed several
times with 95% ethanol, and finally washed
with absolute ethanol. The acid-precipitated
85 protein fraction is then plated out on planchets
(metal discs), dried, and the radioactivity
counted in a gas flow counter.

A third method for evaluating the viability
of cells is the cellular respiration technique.
90 This method is based on the standard War-
burgh manometric respiration technique
(*Manometric Techniques* by W. W. Umbreit,
R. H. Burris and J. F. Stauffer, Burges
Publishing Company 1957) using 10^{-3} molar
95 glucose as a substrate. In this assay, the
oxygen consumption of the cells as they
respire is measured by means of a special
manometer. The amount of oxygen consumed
is measured over a period of time and indi-
cates whether or not the cells are viable by
their ability to respire as compared to control
cells.

The use of cell cultures for the cultivation
of viral agents is essential in the production
of either live-virus or killed-virus vaccines
for human use.

A virus is an extremely small agent which
100 multiples in living cells. Unlike micro-
organisms, viruses cannot be cultivated in
synthetic media. Since living cells removed
from the animal body lose their capability for
carrying out the normal biological functions
105 very rapidly, the preparation of cell or tissue
cultures is essential for the propagation of a
virus if a host organism cannot be used.

One important example of a virus vaccine
is poliomyelitis vaccine, the Sabin type being
an example of a live-virus vaccine and the
Salk type containing killed organisms. In the
preparation of these vaccines the virus is
cultivated in cell or tissue cultures. For ex-
ample, cultured cells obtained from the cortex
of *Rhesus* or *Cynomolgus* monkey kidneys
are commonly used in poliomyelitis vaccine
125 manufacture.

One of the advantages of the process of this
invention is that whole or minced organs can
be frozen and then stored at very low tem-
peratures for long periods of time while main-
130



illustrative



aluminum

maintaining a high animal percentage of viable cells. Thus, the animal is sacrificed in or near its home and then the frozen tissue can be transported under suitable refrigeration to any country where the tissue is needed for production. Not only is the difficulty of transporting and handling animals eliminated, but the problems connected with preservation and transport of suspensions under refrigeration conditions are avoided by the inherently simpler procedure of transporting frozen whole or minced organs. In addition, the complex cell separation procedures do not have to be carried out in an environment where conditions may be less favorable. Furthermore, stockpiling of preserved tissue to permit a flexible vaccine production schedule is an additional advantage of this invention.

The following illustrative examples are presented.

EXAMPLE 1.

Two samples were employed in this example, one being subjected to the freezing, storage and thawing steps of the process of this invention and the other serving as an unfrozen control.

Preparation of Control Sample.

A rabbit was sacrificed and the renal circulation isolated as previously described. The kidneys were perfused at a rate of 1 ml/min. with a total of 50 ml. of perfusion fluid of the following composition (volumetric basis):

- 10% egg yolk
- 20% glycerol
- 20% rabbit serum
- 50% Eagle's MEM

After perfusion was completed, the kidney was removed and the cortical tissue was minced. The minced tissue was trypsinized four times with a 0.25% trypsin solution, with the initial yield of cells being discarded in order to eliminate the cytotoxin. Finally, the trypsinized cells were washed twice with GKN solution containing 10% rabbit serum and resuspended in 25 ml. of growth medium. Fifty replicate tubes containing 5×10^5 cells per milliliter were prepared and incubated at 37° C.

Preparation of Test Sample.

The procedure through the perfusion and mincing steps was identical to that employed with the control sample. The minced tissue was suspended in 10 ml. of the above-described perfusion fluid in a cylindrical aluminum container and cooled for 5 minutes until the minced tissue-perfusion fluid combination had solidified. It was thereafter further cooled in a controlled rate freezer at a rate of approximately 1° C. per minute until the tempera-

ture was placed in a refrigerator of from immediately thawed trypsin that used tubes containing 5×10^5 cells per milliliter were prepared and incubated at 37° C.

Results.

Growth curves comparing the growth obtained for the sample subjected to the freezing and thawing procedure with that obtained with the control sample were computed by conventional methods. The growth curves showed that the test sample grew at about the same rate as the control sample and attained about the same maximum cell concentration.

EXAMPLE 2.

Six preparations of monkey kidney cells, designated Samples 1 to 6, were obtained by the methods described in detail hereinabove. The perfusion medium employed in obtaining Samples 1 to 6 had the following composition:

- 10% egg yolk
- 20% calf serum
- 10% dimethylsulfoxide
- 60% Eagle's MEM

Sample 1—The kidney was perfused *in situ*, excised, cooled for 9 minutes until the kidney and perfusion fluid had solidified, frozen whole to a temperature of -50° C. at a rate of 0.8° C. per minute, stored in liquid nitrogen vapor (about -170° C.) overnight, thawed rapidly in a 37° C. bath, minced, and trypsinized. The trypsinization was carried out using 0.19 per cent trypsin in buffered solution at room temperature for two successive one hour periods.

Sample 2—Same procedure as Sample 1 except that the trypsinization was carried out using 0.2 per cent trypsin in buffered solution at room temperature for an initial period of ten minutes and then for three successive forty minute periods.

Sample 3—The kidney was perfused *in situ*, excised, cooled for 9 minutes until the kidney and perfusion fluid had solidified, minced, frozen to a temperature of -50° C. at a rate of about 1.3° C. per minute, stored in liquid nitrogen vapor (about -170° C.) overnight, thawed rapidly in a 37° C. bath and trypsinized, the trypsinization being carried out as for Example 1.

Sample 4—Same procedure as Sample 3 except that the trypsinization was carried out as for Sample 2.

Sample 5—The kidney was perfused *in situ*, excised, minced and trypsinized as for



Sample
non-

5 minced and
sample served as a
control.

10 For each of the six preparations, a viable count was made using vital staining technique. Both bottle and tube cultures were then planted at a standard level (1.5×10^5 cells/ml. for test tubes and 7.5×10^4 cells/ml. for bottles) and observed periodically. On the seventh day, groups of tubes from each of the six preparations were prepared for testing for virus sensitivity by comparing titers. (Virus sensitivity refers to the ability of the cell culture to support the growth of a particular type of virus. The titer is equal to the negative logarithm of the dilution factor for the lowest concentration at which the culture will propagate the virus, that is, if the virus preparation could be diluted to a concentration of 10^{-7} times the original value and growth would still occur then the titer would be 7).

Also on the seventh day both bottle and tube cultures were inoculated with myelitis, adenovirus and measles virus. This harvest was at estimated optimal yield and titered on a standard strain to give comparative virus yield.

In addition to the evaluation of virus sensitivity and growth, uninoculated cultures were transferred from the growth medium to maintenance medium on the seventh day to evaluate the long-term maintenance of cell cultures prepared from preserved kidney tissue. (A maintenance medium is a nutrient medium capable of maintaining the cell metabolism but not of supporting growth.) Observation over an extended period of all kidney cell cultures intended for virus propagation is a standard precaution taken to ensure that a latent virus is not present in the cells.

Table I presents qualitative observations describing the cell cultures in growth medium and in maintenance medium as well as virus titers indicating virus sensitivity.

TABLE I

Sample No.	Viable Cells Obtained per kidney after trypsinization $\times 10^6$ (1)	Evaluation of cell sheet After 7 days growth (2)		Virus Titers on Tube Cultures (3)			Quality of (4) Tube Cultures (14 days)		Quality of Boule Cultures (4)	
		Completeness	Quality	Polio	Adeno-	Measles	(14 days)		14 days	28 days
1	54	++ light (5), but O.K. for general use	++ clean, a few atypical cells	7.33	—	—	Fair		Light, O.K.	Light, good
2	35	+ too light for general use	+ clean, frequent giant cells	6.66	—	—	Poor		Poor	Very light, O.K.
3	114	++++ very good	++++ granular	7.5	3.63	3.33	Good		Good	Light, good
4	75	+++ good	+++ granular	6.83	3.5	2.66	Fair to poor		Light, O.K.	Light, O.K.
5	109	++++ very good	++++ granular	7.5	4.38	3.5	Good		Good	Good
6	180	++++ good, but with rounded cells	++++ granular, foamy areas	7.5	4.0	3.66	Good		Good	Good

Notes:

- (1) Based on Trypan Blue staining. All suspensions showed 85—90% viable. Differences in number of viable cells is due to different size difference in degree of trypsinization, and formation of cell fragments due to freeze-thaw damage.
- (2) Completeness refers to the formation of confluent cell layers. Quality refers to appearance of individual cells (++++ indicates optimal). The term "clean" means that there is an absence of granules. The quality of cell growth was essentially the same in both tubes and bottles.
- (3) The numbers presented here provide a comparison between test samples and control samples.
- (4) These observations refer to the appearance of the cultures that were transferred from growth medium to maintenance medium on the 7th day.
- (5) The term "light", as used in this Table, means that the cell sheet is not contiguous.

Table six relative yields from bottle cultures for of the

TABLE II
Yields on Bottle Cultures

Sample No.	Adeno-		Adeno-		Adeno-	
	16-oz.	28-oz.	16-oz.	28-oz.	16-oz.	28-oz.
1	7.17	—	2.50	—	2.50	—
2	6.50	—	—	—	—	—
3	7.17	7.50	3.38	—	2.63	—
4	7.17	—	3.16	—	2.83	—
5	—	7.63	—	3.83	—	3.5
6	7.50	7.63	4.16	—	2.83	—

5 This example shows that the process of this invention can provide preserved tissue cell aggregates containing a high yield of viable cells capable of supporting virus growth.

EXAMPLE 3.

10 The data presented in this example illustrate the importance of cooling the frozen cell aggregates from completion of the phase change to -50°C . at a rate no greater than 3°C . per minute.

15 In this example rabbit kidneys were perfused in situ employing a perfusion medium having the following composition:

20% rabbit serum
10% dimethylsulfoxide
70% Eagle's MEM

Each kidney was minced, suspended in the perfusion medium, cooled from the freezing point through the phase change in a period of 2 minutes and then cooled to a temperature of -50°C . at the rates set forth in Table III. The samples were then cooled rapidly to liquid nitrogen temperatures, thawed rapidly in a water bath at 37°C . trypsinized and cultured by conventional procedures. The growth rate data for the various rates of cooling are presented in the following Table III. In the table, each cell concentration reading is the average of four culture tubes.

TABLE III

Viable Cell Concentration (cells/ml.)

Growth Period (Days)	Freezing Rate ($^{\circ}\text{C}/\text{min.}$)			
	0.96	3.1	4.1	8.0
0	5.4×10^5	6.2×10^5	5.0×10^5	5.2×10^5
4	—	7.3×10^4	—	—
6	1.5×10^5	2.5×10^5	4.3×10^4	—
8	8.8×10^5	1.0×10^6	8.7×10^4	—
9	1.0×10^6	—	—	—
10	—	—	2.9×10^5	2.2×10^4
12	—	—	3.8×10^5	—
13	—	—	—	4.1×10^4

With f
min. t

5

conc
7 day

10

rates of 0.96 and 3.1° C./
cultures grew to cell concentra-
t 1×10^6 cells/ml. in 8 to 9
r 4.1 and 8.0° C./min. freezing
had not reached this con-
to 13 days. Cultured cells
control kidneys grew to a
about 1×10^6 cells/ml. in

the
cer

per

20

The point cooled to the freezing
point from the freezing
point the change in a period
of 2 minutes cooled to liquid nitrogen
temperatures, and warmed to room tempera-
ture under substantially identical conditions.
The additive was removed by washing. The
viability of the frozen and thawed cells was
then compared with control samples which
had not been cooled.

25

30

15 In this example, three samples of rabbit
bone marrow were suspended in media having

The results are summarized in Table IV.

TABLE IV

Cooling rate (room temperature to freezing point)	Viability as % of control (cellular respiration method)
1° C./min.	74
3—4° C./min.	69
8° C./min.	72



EXAMPLE 5.

the

35

This example shows that the time which
the cells spend in liquid solid phase change
should be less than 10 minutes and preferably
as short as possible.

40

Three samples of rabbit bone marrow
(Group A) were suspended in media having
the composition (volume per cent):

70% Hank's medium
15% dimethylsulfoxide
15% rabbit serum

45

and three additional samples of rabbit bone
marrow (Group B) were suspended in media
having the composition (volume per cent):

70% Hank's Medium
15% glycerol
15% rabbit serum

50

Each group of samples was cooled to the
freezing point at a rate of 1 to 2° C. per

minute, cooled through the phase change in
varying periods of time and then cooled to
-50° C. at 1° C. per minute, cooled rapidly
to liquid nitrogen temperatures, and thawed
in a water bath at 37° C. and viability com-
pared with unfrozen control samples. The
data are summarized in Table V. Viability
of cells in Group A samples was measured
by the C^{14} -glycine incorporation method and
viability of cells Group B samples was
measured by the cellular respiration method.
These two viability assays have been shown
to correlate with each other and with an
irradiated animal assay. The viabilities are
somewhat lower than in the previous example
due to the inhibitory effect of the additive
present. The data based on two assay methods
and two additives show that the shortest heat
of fusion period is better than the longer
periods for optimum viability.

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65

70

TABLE V

Duration of phase change (min.)	Viability as % of Control	
	Group A	Group B
1 — 2	52	45
8 — 10	35	33
15 — 25	2	3

This
 port
 through
 5 as possible. The
 were obtained by the
 lined hereinabove.
 perfused with a perfus
 10 2.5 wt.% glucose
 0.6 wt.% methylcellulose (15 centi-
 poise viscosity)
 20% rabbit serum
 16.9% water
 10% dimethylsulfoxide
 15 50% Eagle's MEM

Before use, the perfusion medium was
 stirred for 1 hour in an atmosphere com-
 posed of 95 volume per cent oxygen and 5

volume per cent carbon dioxide
 of 15 psig. (pounds per square
 The kidneys were then min
 dly pended in the perfusion me
 neys samples of suspended rabbit
 out- cooled to the fusion temper
 were C. per minute, cooled the
 lining: change in varying periods of
 to
 -50° C. at 1° C. per min
 25 cooled to liquid nitrogen tempera
 to
 in a water bath at 37° C., cultured and the
 growth rate compared with unfrozen control
 30 samples. The culture media were inoculated
 with cells at a concentration of 5×10^5
 cells/ml. The unfrozen control reached a con-
 centration of 1×10^6 cells/ml. in seven days.
 The growth rate data for the cultures obtained
 35 from the frozen rabbit kidney cells are sum-
 marized in Table VI.

TABLE VI

Viable Cell Concentration (cells/ml.)

Growth Period (days)	Duration of Phase Change (min.)			
	2	4	8	16
0	5.4×10^5	5.0×10^5	5.0×10^5	5.2×10^5
4	1.1×10^5	6.6×10^4	—	—
5	2.5×10^5	—	—	8.1×10^4
6	5.7×10^5	—	7.6×10^4	—
7	1.1×10^6	8.7×10^4	—	4.7×10^5
8	1.3×10^6	3.9×10^5	3.2×10^5	1.0×10^5
9	1.4×10^6	4.0×10^5	—	—
10	—	—	1.6×10^6	1.2×10^5
11	—	1.5×10^6	1.8×10^6	4.8×10^5
12	—	1.7×10^6	1.4×10^6	1.1×10^6
13	—	—	1.5×10^6	9.8×10^5

40 The data in Table VI shows that where the
 cells were cooled through the heat of fusion
 period in two minutes the rate of growth
 was approximately equal to that of unfrozen
 controls. Where the time in heat of fusion was
 4 to 8 minutes, growth to a cell concentration
 45 of 1×10^6 cells/ml. was delayed until about
 the tenth day. Where the cells spend 16
 minutes in the heat of fusion period, the rate

of growth was substantially retarded.

A comparison of Examples 5 and 6 shows
 that the importance of cooling cells rapidly
 50 through the heat of fusion period is demon-
 strated equally well by three different criteria
 of viability; namely C^{14} -glycine incorporation,
 cellular respiration, and period of time for
 growth of cultured cells from 5×10^5
 55 cells/ml. to 1×10^6 cells/ml.

EXAMPLE 7.

This illustrates the importance of back pressure during perfusion of whole rabbit kidneys were perfused outlined above and *in situ* with a perfusion medium having compositions:

5% glucose
1% methylcellulose (15 centipoise viscosity)
20% rabbit serum
16.9% water
10% dimethylsulfoxide
50% Eagle's MEM

Before use, the perfusion medium was stirred for 1 hour in an atmosphere composed of 95 volume per cent oxygen and 5 volume per cent carbon dioxide at a pressure of 15 psig.

Both kidneys were completely perfused but in one kidney a vein was cut so that there was essentially no back pressure during the perfusion operation. Both whole kidneys were placed in aluminum containers and were cooled to the fusion temperature at 1° C. to 2° C. per minute, cooled through the phase change in a period of 2 minutes, cooled to -50° C. at an average rate of 1° C. per minute, quickly frozen to liquid nitrogen temperatures, thawed in a water bath at 37° C., minced, trypsinized and cultured at an initial cell concentration at 5×10^5 cells/ml. The cells from the kidney perfused with back pressure reached a cell concentration of 1.4×10^6 cells/ml. in 9 days. The cells from the second kidney, perfused without back pressure, grew very poorly and reached a maximum cell concentration of only 4×10^4 cells/ml.

EXAMPLE 8.

This example illustrates the methods of this invention as applied to the preservation of rat lung.

Rat lung was perfused *in situ* with a medium having the following compositions:

2.5 wt.% glucose
0.6 wt.% methylcellulose (15 centipoise viscosity)
20% fetal calf serum
16.9% water
10% dimethylsulfoxide
50% Eagle's MEM

The perfusion medium was stirred for 1 hour in an atmosphere composed of 95 volume per cent oxygen and 5 volume per cent carbon dioxide at a pressure of 15 psig. prior to use.

The perfused lung was minced, suspended in perfusion medium, cooled to the freezing point, cooled through the phase change in about 6 minutes, further cooled to -50° C. at 1 to 2° C. per minute, further cooled to liquid nitrogen temperatures and then thawed

by lung cell concentration. The frozen lung was thawed by the process outlined above. The frozen lung at an initial concentration of 1×10^5 cells/ml. grew to a concentration of 1×10^6 cells/ml. in 10 days. Control cells from a rat lung subjected to identical treatment except for the freezing and thawing steps grew from an initial concentration of 5×10^5 cells/ml. to a concentration of 1×10^6 cells/ml. in about 9 days.

EXAMPLE 9.

This example illustrates a preferred trypsinization procedure especially suitable for use in trypsinizing tissues, particularly minced tissues, which have been frozen in accordance with the practice of this invention.

Trypsinization Solution.

The trypsinization solution employed in this Example is prepared as follows:

A 10 weight per cent solution of trypsin was produced by adding trypsin powder to an aqueous solution containing the following materials:

Methyl cellulose (15 cps)	6.0 grams per liter
NaCl	8.0 grams per liter
KCl	0.4 grams per liter
Na ₂ HPO ₄	0.06 grams per liter
KH ₂ PO ₄	0.06 grams per liter
Glucose	50.0 grams per liter

The mixture so formed was stirred with a magnetic stirrer for 10 minutes at 4° C. and then, centrifuged at 1000 revolutions per minute at 4° C. The supernatant liquid was decanted and diluted with 50 equal volumes of aqueous solution containing the following materials:

NaCl	8.0 grams per liter
KCl	0.04 grams per liter
Na ₂ HPO ₄	0.06 grams per liter
KH ₂ PO ₄	0.06 grams per liter
NaHCO ₃	0.5 grams per liter

The final diluted solution was filtered in order to sterilize it, and the filtrate so obtained (contained 0.2 weight per cent trypsin) was used as a trypsinization solution as described below.

CELL GROWTH MEDIA.

The cell growth media employed in this Example and in the following example contained from 2 to 40 volume per cent of calf serum, 0.5 weight per cent lactalbumin hydrolysate, 200 micromoles of glutamine, and from 60 to 98 volume per cent of Eagle's MEM (defined above).



aluminum

concentration

- used. An minced tissue is maintained at 37° C. for inversion of the tube and thawing of the minced tissues are decanted into an indented trypsinization flask and a portion (e.g. 200 milliliters) of the above-described trypsinization solution is added to the flask. The tissue is then trypsinized by stirring the contents of the flask for about one hour, employing a magnetic stirrer at the outset of the stirring, the contents of the flask are at 4° C. and at the end of the hour they have warmed to about room temperature. The stirring is at a rate sufficient to cause good agitation of the tissue-solution mixture but not so great as to cause frothing of the trypsinization solution. The untrypsinized tissues are allowed to settle and the supernatant liquid containing the trypsinized cells is decanted through gauze. If trypsinization is incomplete, another portion (e.g. 200 milliliters) of the trypsinization solution is added to the flask containing the untrypsinized tissues and the trypsinization is repeated (e.g. for 0.5 to 1.0 hours) until trypsinization is complete. The contents of the flask are allowed to settle and the supernatant liquid containing the trypsinized cells is decanted through gauze. A portion (or portions) of supernatant liquid is centrifuged through gauze is centrifuged at 600 revolutions per minute for 15 minutes. The supernatant liquid from the centrifugation is decanted and the cell wash is decanted during the centrifuging and 10 to 20 milliliters of the described growth medium containing 10 percent calf serum and 90 volume percent Eagle's MEM. The resuspended cells are again centrifuged at 600 revolutions per minute, the supernatant liquid is decanted and the cells which had separated are again resuspended in 10 to 20 milliliters of growth medium. The yield and viability of the latter resuspended cells is determined by the vital staining method described above. The resuspended cells are stored at 4° C. till used (e.g. as described in Example 10).
- Results.
- The following table summarizes the results obtained when minced monkey kidney tissues and minced rabbit kidney tissue, which had previously been frozen in accordance with the practice of this invention, were thawed and trypsinized in accordance with the above procedure. For comparison purposes, the table shows results obtained when unfrozen minced monkey kidney tissue and rabbit kidney tissue were similarly processed.

RESULTS OF TRYPSINIZATION
Trypsinization

Animal	Prior Treatment	Tissue	Time	Solution	% Viability	% Trypsinization	Cell Yield (2)
Cynomolgus Monkey	Unfrozen	Cortex	1.5 hrs.	200 ml.	92 (1)	64.5 (1)	6.7×10^7 (1)
Cynomolgus Monkey	Frozen DMSO + O ₂ (3)	Cortex	1 hr. 0.5 hr.	200 ml. 200 ml.	76.5 (1)	*50-80 (1)	6.9×10^7 cells per kidney
Rhesus Monkey	Frozen DMSO + O ₂ (3)	Cortex	1 hr. 0.5 hr.	200 ml. 200 ml.	75	<50	4.1×10^7 cells per kidney
Rabbit	Unfrozen	Cortex	1 hr.	100 ml.	89	—	5.4×10^7
Rabbit	Frozen DMSO + O ₂ (3)	Cortex	1 hr.	100 ml.	87.5 (1)	73.5 (1)	7.9
Rabbit	Unfrozen	Whole	1 hr.	100 ml.	88	—	—
Rabbit	Frozen DMSO + O ₂ (3)	Whole	1 hr.	100 ml.	88 (1)	76 (1)	9.

(1) Average of two runs.

(2) Yield of cells per gram of frozen tissue, unless otherwise noted.

(3) Minced tissue frozen in accordance with the process of this invention after treatment with 10 weight per cent dimethylsulfoxide solution saturated with oxygen and also containing serum, Eagle's MEM and methyl cellulose.

* Estimation.

It is critical to obtain high per cent cell growth when the stirring procedure is varied significantly (e.g. when this stirring was conducted for 12 hours at room temperature), drastically reduced per cent cell viability was observed. It is also highly desirable to stir the tissue-trypsinization solution mixture near but below the rate of stirring at which frothing occurs.

EXAMPLE 10.

This example illustrates the improved growth of cells achieved when the preferred trypsinization procedure described in Example 9 is coupled with the preferred cell growth procedure described below.

Cell Growing Procedure.

Portions of resuspended cells as obtained in Example 9 cells are diluted to a concentration of 5×10^5 cells per milliliter and then are added to both standard 12.5 centimeter $\times$ 15 millimeter test tubes and to standard 8 ounce prescription bottles. One milliliter of diluted resuspended cells is added to the tubes and 10 milliliters to the prescription bottles. Duplicate runs were made in the tubes and bottles to determine if the container configurations had any effect on cell growth. No effect due to container configuration was observed.

A stream of a gaseous mixture consisting of 5 volume per cent carbon dioxide and 95 volume per cent of air was brought in contact with the surface of the cell suspensions for a short period of time, e.g., from 1 to 4 seconds. The gaseous mixture was drawn from pressurized tanks (pressure in the tanks were from 200 to 2200 psig.). The mixture was conducted from the tanks to the surfaces of the suspensions through a filter ($6\frac{1}{2}$ inches long and 1 inch in diameter) and then through a diameter tube (1 inch long and $1/16$ inch diameter) and had a considerable velocity on contact with the surfaces of the suspensions. The tubes and bottles were then sealed and incubated at 37° C. In the case of the tubes, the original growth media was replaced with fresh growth media every 48 hours. This was not required in the bottles since the media therein was not depleted appreciably during the cell growth period.

RHESUS MONKEY

Three suspensions of Rhesus kidney tissue which had been trypsinized in Example 9 were subjected to the described cell growing procedure in growth media containing 10 volume per cent calf serum and 10 volume per cent Rhesus monkey kidney serum. Other Rhesus monkey kidney suspensions were prepared similarly. The surfaces of the suspensions were not brought into contact with the carbon dioxide-air mixture. After 24 hours, the cell suspensions that had been treated with the carbon dioxide-air mixture exhibited better attachment to the inner walls, the glass containers and better growth than the other (non-gassed) cell suspensions. In addition, the cell suspensions that had been treated with the carbon dioxide-air mixture had a pH of 7.1 to 7.3 whereas the cell suspensions that had not been so treated had a pH of 7.6 to 7.8. After 6 to 7 days of incubation complete confluent sheeting of the cell suspensions that had been treated with the carbon dioxide-air mixture was noted on the container walls. No further growth and no sheeting of the cell suspensions that had not been treated with the carbon dioxide-air mixture was noted. After 8 days the cell count in the tube containing the suspension having 10 volume per cent serum that had been treated with the carbon dioxide-air mixture was 1.3×10^6 cells per milliliter.

CYNOMOLGUS MONKEY TISSUE

The two frozen Cynomolgus monkey kidney tissue suspensions prepared as described in Example 9 were combined and cultured in both tubes and bottles in cell growth media containing serum concentrations of 2 volume per cent, 5 volume per cent and 10 volume per cent serum. The tubes and bottles were the same as described above. These samples were subjected to the cell growing process described above. Three other samples of the combined suspensions were similarly treated except that they were not contacted with the carbon dioxide-air mixture. After 24 hours of incubation, attachment of cells to the container inner surfaces was noted in all of the containers but there was more attachment in the containers where the cell suspensions had been contacted with the carbon dioxide-air mixture. After 6 days of incubation, confluent sheeting on the container inner surfaces was noted in all of the containers but greater cell growth was observed where the cell suspensions had been treated with the carbon dioxide-air mixture. The yields of cells in the tubes were as follows:



Carbon Concentration (vol.—%)	Yield of Tissue Cells Treated with CO ₂	
2	1.1×10^6	10^4
5	1.1×10^6	10^4
10	1.3×10^6	2.2×10^4

The latter yields indicates the desirability of contacting the cell suspensions with a stream of an air-CO₂ mixture, prior to growth. Apparently carbon dioxide is absorbed by the suspensions from such mixtures and retards undesirable increases in the pH of the suspensions above 7.3 during cell growth. In general, it is preferred to contact the cell suspensions with streams of air-CO₂ mixtures containing from 2 to 20 volume per cent CO₂ and from 80 to 98 volume per cent air. Alternately, good results can be achieved by using conventional buffers to prevent the pH rising above 7.3 during the growth of the cells. The pH should also be no lower than 7.0.

WHAT WE CLAIM IS:—

1. A process for preserving animal tissue cells which comprises (1) contacting such cells with a protective additive which reduces cell damage during subsequent freezing, (2) cooling the cell-protective additive combination to freezing point and then making the liquid to solid phase change in less than 10 minutes, latent heat of fusion being removed, (3) further cooling the solidified cells to -50° C. at a rate of not more than 3° C. per minute, and (4) further cooling the frozen cells and maintaining the frozen cells at a temperature below -75° C.

2. A process as claimed in claim 1, in which the protective additive is glycerol, ethylene glycol, an aldose, a ketose, a polysaccharide, dextran, polyvinylpyrrolidone, albumin isolated from serum, dimethyl sulfoxide, mannitol, urea, or mixtures thereof.

3. A process as claimed in claim 1 or 2, in which the protective additive is employed in a liquid medium which includes nutrients for cells.

4. A process as claimed in claim 3, in which the nutrients are amino acids, saccharides, inorganic salts, proteins, vitamins and autologous, homologous or heterologous animal serum.

5. A process as claimed in any of claims 1 to 4, in which the frozen cells are maintained at a temperature below -75° C. using liquid nitrogen.

6. A process as claimed in any of claims

1 to 5, in which the frozen cells are maintained at a temperature below -130° C.

7. A process as claimed in claim 6, in which the liquid to solid phase change of the combination of cells and additive is made in 2 minutes or less.

8. A process as claimed in any of claims 1 to 7, in which the cells are in the form of an organ.

9. A process as claimed in claim 8, in which the organ is perfused under pressure with a perfusion medium containing nutrients for the cells of the organ and a protective additive, prior to freezing.

10. A process as claimed in claim 9, in which the organ is minced after perfusion and the mince suspended in the perfusion medium prior to freezing.

11. A process as claimed in any of claims 1 to 7, in which the cells are in the form of bone marrow.

12. A process as claimed in claim 1 in which the tissue cells are monkey kidneys that have been first perfused under sufficient back pressure to cause turgidity and distension of said kidney with a medium having one of the following compositions (in volume per cent unless otherwise noted):

(A) 10% egg yolk
20% calf serum
20% glycerol
50% Eagle's MEM as hereinbefore defined

(B) 10% egg yolk
20% calf serum
10% dimethylsulfoxide
60% Eagle's MEM as hereinbefore defined

(C) 2.5 Wt.% glucose
0.6 wt.% methylcellulose (15 centipoise viscosity)
20% calf serum
16.9% water
10% dimethylsulfoxide
50% Eagle's MEM as hereinbefore defined



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13. which perfused medium.
- 5 14. A process in which the tissue cell marrow that is first having one of the following (in volume per cent)
- 10 (A) 70% Hank's medium as hereinbefore defined
15% glycerol
15% rabbit serum
- 15 (B) 70% Hank's medium as hereinbefore defined
15% dimethylsulfoxide
15% rabbit serum
- 20 15. A process as claimed in claim 1 substantially as hereinbefore described with reference to any of the Examples.
- 25 16. Frozen cells whenever prepared by a process as claimed in any of claims 1 to 15.
- 30 17. A process for trypsinizing thawed cells which had been frozen at a temperature below -75°C . by a process as claimed in claim 1, which comprises conducting the trypsinization in a trypsinization solution for a period of from 1 to 17 hours at a temperature from 30°C . to 4°C . while stirring the solution near but below the rate of stirring at which frothing occurs.
- 35 18. A process as claimed in claim 17, in which the thawed cells are prepared by thawing frozen cells as claimed in claim 16.
19. A process as claimed in claim 18, in which the thawing is achieved by means of a warm water bath.
20. A process as claimed in claim 19, in which the water bath is maintained at a temperature of 37°C .
21. A process as claimed in claim 20, substantially as hereinbefore described with reference to any of Examples 1, 2, 3, 5, 7, 8 and 9.
22. Trypsinized thawed cells whenever produced by a process as claimed in any of claims 17 to 21.
23. A process for growing thawed cells which had been frozen at a temperature below -75°C . by a process as claimed in claim 1, which comprises maintaining the cells at a pH from 7.0 to no greater than 7.3 during growth.
24. A process as claimed in claim 23, in which the thawed cells are prepared by thawing frozen cells as claimed in claim 16.
25. A process as claimed in claim 24, in which the thawing is achieved by means of a warm water bath.
26. A process as claimed in claim 25, in which the water bath is maintained at a temperature of 37°C .
27. A process as claimed in claim 23 substantially as hereinbefore described with reference to any of Examples 1, 2, 3, 5, 7, 8 and 9.
28. Grown thawed cells whenever produced by a process as claimed in any of claims 23 to 27.

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