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<p>(54) Title: ANTI-INVASION FACTOR CONTAINING CULTURES</p>		
<p>(57) Abstract: Chondrocytes are cultured to produce cartilage tissue from which an anti-invasion factor (AIF) may be recovered. Mammalian cartilage providing at least about 60% viable chondrocytes is enzymatically digested to denude the cells of their extracellular matrix. The denuded cells are plated at high density and cultured to produce cartilage tissue.</p>		

The Department of International Law, Ministry of Foreign Affairs of the Netherlands, is pleased to announce that it has published the Yearbook of International Law for 1980-1981. This volume contains the text of the Convention on the Granting of Patents to Inventors, signed at Washington on June 19, 1979, and the text of the Protocol to the Convention, signed at Washington on June 19, 1979. The Yearbook also contains the text of the Convention on the Granting of Patents to Inventors, signed at Washington on June 19, 1979, and the text of the Protocol to the Convention, signed at Washington on June 19, 1979. The Yearbook is available in Dutch and English. The price of the Yearbook is 100,- Dutch Guilders. The Yearbook is available from the Department of International Law, Ministry of Foreign Affairs, The Hague, The Netherlands.

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ANTI-INVASION FACTOR CONTAINING CULTURES

The invention described herein was made in the course of work under a grant or award from the Department of Health, Education and Welfare.

The present invention relates to culturing of cartilage cells in vitro to produce cartilage tissue. The resultant tissue and its growth medium has utility as a source of a protein fraction having anti-invasive properties.

Certain tissues have a high resistance to invasion by foreign cells, and cartilage tissue has been found to have especially high resistance to invasion by foreign cells such as cancer cells and blood capillary cells.

Described in my U.S. Patent No. 4,042,457, issued August 16, 1977, is a protein fraction obtained from cartilage tissues which inhibits cell proliferation and tissue invasion. This protein fraction was also found to have inhibitory effects on bone resorption as described in my U.S. Patent No. 4,176,177, issued November 27, 1979. This protein fraction will hereinafter be referred to as anti-invasion factor, or AIF.

AIF has been shown to express anti-tumor properties both in vivo and in vitro. Tumors grown in vivo shrink in the presence of AIF. Tumorous mice injected with sufficient AIF demonstrate regression of the tumors and a lack of new daughter tumors. Accordingly, AIF holds promise as a cancer-treating agent. However, AIF must be made more readily obtainable for its full drug potential to be realized.

Current methods of obtaining AIF involve obtaining waste animal parts from slaughterhouses, separating the cartilage tissue and extracting the AIF. This is a long and tedious procedure. Cartilage tissue

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is a part of the skeleton of animals and, accordingly, bones and adjacent muscles and other connective tissues must be separated to reach the cartilage to remove the small amount of cartilage tissue therefrom.

5 As a first step toward increasing the available amount of AIF, it would be desirable to obtain cartilage tissue by means other than separating animal joints, such as by culturing the cartilage cell population, chondroblasts, or chondrocytes to obtain cartilage
10 tissue having significant amounts of AIF.

 Culturing of cartilage cells containing significant amounts of AIF is complicated by the inherent nature of cartilage tissue. Cartilage cells in vivo produce an extracellular matrix which separates one
15 cell from another. One of the main functions of the cells comprising cartilage would appear to be the production of the extracellular matrix. These cartilage forming cells are called chondroblasts. While an animal is growing, the cartilage is growing and many cartilage
20 cells retain their ability to reproduce.

 In an adult animal, the majority of cartilage cells are embedded within the extracellular matrix and are non-reproductive and responsible for the maintenance of the tissue integrity. Even though functionally
25 incorrect, all cartilage cells, whether reproductively active or inactive, are generally called chondrocytes. The extracellular matrix, comprised largely of collagen type II and proteoglycans is extremely long lasting, cartilage collagen in certain animals may have a
30 half-life of several years. Accordingly, when the chondrocytes have produced the extracellular matrix around themselves and thereby produced the cartilage tissue, they have generally served their function. Chondrocytes from adult cartilage tissue have generally
35 lost their in vivo potential to reproduce.

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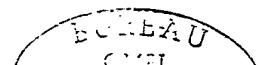
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The most actively reproducing chondrocytes are those found in embryonic tissue. However, embryonic tissue appears to contain less anti-invasion factor than adolescent tissue. Illustrative of this fact is that while adult cartilage tissue is nonvascular, as a result of the anti-invasion factor preventing the ingrowth of capillary cells, some early embryonic cartilage is supplied by the vascular system.

A further well recognized difficulty in culturing cartilage tissue is the fact that chondrocytes do not necessarily produce cartilage tissue when cultured.

When chondrocytes obtained from most cartilage tissue are cultured, the resulting tissue includes fibroblasts, that is, cells which have not differentiated into cartilage producing cells or modulated chondrocytes with fibroblastic gene expression. Such cultures produce collagen type I, the most common type of collagen which is found in tissues such as skin, bone, muscle, etc., as well as collagen type II, the type of collagen found in cartilage. In order to produce true cartilage tissue, therefore, it is necessary that the chondrocytes maintain their differentiated state and proliferate only into cartilage type cells with an extracellular matrix therearound containing only collagen type II. While collagen type II would appear not to be a component of AIF, the production of collagen type II is a good indicator of the presence of AIF as both collagen type II and AIF are closely associated with cartilage tissue.

It has been recognized that relative amounts of collagen type I and collagen type II which have been obtained from chondrocyte cultures depend on the environment surrounding the cultured chondrocytes. Different cellular environments give rise to cultures



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with collagen type I and collagen type II in varying amounts. An extremely significant environmental factor has been shown to be the density in which the chondrocytes are suspended and plated. For uncertain reasons, chondrocytes resist contact with other chondrocytes and when chondrocytes are in contact with other such cells, they produce around themselves the extracellular matrix found in cartilage. Studies with embryonic chicken chondrocytes have shown that when the chondrocytes are plated in very high density, primarily collagen type II is produced. Likewise, it has been found in fetal bovine cartilage tissue that a high density of plated chondrocytes yields a high percentage of collagen type II as compared to collagen type I.

The plating of mature mammalian chondrocytes to produce cartilage tissue heretofore has been less than completely successful in that culturing of mammalian tissue has generally produced cultures having a mixture of collagen type I and collagen type II.

In order that anti-invasion factor may be produced in greater amounts it would be desirable to be able to culture mammalian chondrocytes to produce cartilage tissue. To insure sufficient yield of anti-invasion factor, it would be highly desirable to culture chondrocytes to produce cultures with extracellular matrices containing only collagen type II.

Accordingly it is an object of the present invention to culture mammalian chondrocytes to produce cartilage tissue with high amounts of anti-invasion factor.

The objects are achieved in the present invention by plating, in high density, chondrocytes obtained from mammals which have a high percentage of viability so that, generally, each active chondrocyte is in surface contact with another active chondrocyte.

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While it has been recognized that high density plating tends to produce cartilage tissue, previous attempts have been less than successful due to the failure to concentrate reproductively viable cells in sufficient
5 density.

To more fully understand the present invention, the culturing of chondrocytes to produce cartilage tissue will now be described in greater detail.

As hereinabove described, the most active
10 cartilage tissue, i.e. embryonic tissue, is limited in the production of anti-invasion factor, and mature cartilage is metabolically slow and contains cells which are generally inactive, and therefore, when mature tissue is degraded by the best known methods a very low
15 yield of viable chondrocytes is provided. It is therefore desirable to obtain cells from non-embryonic animals which nevertheless provide a high percentage of viable chondrocytes.

It has been found that the chondrocytes of
20 weight bearing articular cartilage as are found in the ankles and toes of adolescent mammals have a very high viability and high degree of biological activity. (The term "adolescent" is used herein in its medical sense to refer to the entire growing period of the animal from
25 birth to maturity.) In particular, calf fetlock cartilage contain a high percentage of reproductively and metabolically active chondrocytes. A growing calf gains weight very rapidly, and to maintain the calf's weight, the weight bearing limbs such as the hooves and
30 fetlocks must be enlarging proportionally to accommodate the increasing weight, and the cartilage tissue is proportionally growing and expanding.

Calf fetlocks are generally a waste product of slaughterhouses and may therefore be obtained with
35 relative ease. After the animal is slaughtered, the



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fetlocks are transported to the laboratory. The skin is removed and the first phalangeal joint is carefully opened under aseptic conditions and the synovial fluid is removed. The cartilage of the exposed joint is
5 carefully shaved off so as to obtain as much cartilage tissue as possible and yet avoid contamination of other tissues such as bone. The shavings are collected in Ham's F-12 medium or any other tissue culture medium suitable for tissue survival but enriched in antibiotics
10 and antifungal agents, i.e. 50 micrograms gentamycin and 5 micrograms of beta-amphotericin per milliliter.

The cartilage shavings contain both the chondrocytes and the extracellular matrix. In order that the cells may be cultured, the extracellular matrix
15 must be removed. This is done enzymatically by various enzymes which digest the proteins of the extracellular matrix. The shavings are first digested with pronase in an amount between about 0.5 and about 2.0% and most preferably about 1% weight per volume in Ham's F-12
20 medium or other suitable tissue culture medium containing 5% bovine serum for 90 minutes. The pronase will digest most of the proteins of the extracellular matrix including proteoglycans and some collagen. Therefore, the shavings after washing and centrifugation
25 are further digested in medium containing 5% bovine serum and between about 0.1 and about 1% weight per volume collagenase for a period of about 1-2 hours to digest the collagen from around the cells. The cells, after washing and centrifugation, are further digested
30 with between about 0.1% and about 3% weight per volume trypsin or testicular hyaluronidase and most preferably about .25% trypsin or hyaluronidase for between about 5 and about 10 minutes in the presence of 5% bovine serum. The trypsin will digest any remaining
35 extracellular proteins to denude the cells. Testicular

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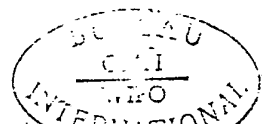
hyaluronidase will digest the sugar moieties attached to any remaining extracellular protein and thereby denude the chondrocytes.

The digestion of the extracellular material by the proteolytic enzymes is very harsh to the cells. The enzymes, in addition to attacking the matrix, begin to attack the cell membranes. Accordingly the cells are left in a weakened state after digestion. Mammalian serum, preferably fetal serum, is therefore added to the digestive mixture so that the denuded cells may be coated and protected with serum immediately after being denuded.

The fact that serum and digestive enzymes can be used simultaneously is somewhat surprising for several reasons. It would be expected that all the trypsin would attack the serum and would bind, for the most part, with proteins of the serum. Furthermore, serum contains trypsin inhibitors which should affect trypsin's action vis a vis the extracellular matrix. Nevertheless, it has been found that this combination of trypsin along with serum produces chondrocytes with a high percentage of viability.

The isolated cells are collected by centrifugation at about 900 rpm for 10 minutes. The cell pellet is washed in phosphate buffered physiological saline pH 7.4 as described in J. Cell Biol. 49: 451, (1971) and the cells are suspended in Ham's F-12 medium without serum. The cells are then passed through a 90 micrometer Nitex screen to separate the cells which are resuspended in Ham's F-12 medium containing about 5-10 percent serum. Sufficient cells are added to the medium to achieve a concentration of at least 10^6 cells per milliliter.

Cells may be grown in culture dishes such as 35 mm tissue culture dishes and for morphological



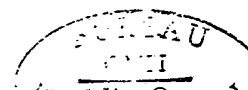
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observation may be grown on 26 x 34 mm cover slips submerged in culture dishes.

In order to produce cartilage tissue, at least a monolayer of denuded chondrocytes must be originally plated. Most of these cells must be reproductively viable in order for growing chondrocytes to contact other growing chondrocytes to produce the extracellular matrix. Accordingly, cartilage tissue which will provide at least about 60% viable chondrocytes, is selected for culturing. Herein, the percent of viable chondrocytes referred to is that percent of chondrocytes which is provided by careful but thorough degradation of the extracellular matrix of a particular tissue.

It has been found that chondrocytes obtained from fetlock cartilage of adolescent bovines may be 89% reproductively viable. This is somewhat surprising in light of the viability of chondrocytes obtained from other tissue such as nasal cartilage from which, because of its easy accessibility, cartilage tissue is commonly obtained. For example, in the same animal where 89% reproductively viable chondrocytes may be obtained from fetlock cartilage degradation, only 10 to 15 percent reproductively viable chondrocytes may be obtained from nasal cartilage degradation. It is therefore possible to plate reproductively viable fetlock-derived chondrocytes in sufficiently high density to produce at least a monolayer of viable cells, such a density being necessary to produce cartilage tissue.

While chondrocytes grown in culture dishes produce the desired tissue, significant amounts of tissue cannot thereby be produced. Culture dishes are small. Furthermore, an important factor in achieving good yields of cartilage tissue is the atmosphere in which the chondrocytes grow. After layers of cells begin to accumulate, the lower layers are shielded from



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the atmosphere. Inability to maintain optimal gas balance for the shielded layers results in reduced production of cultured material.

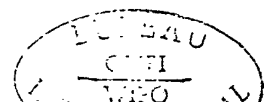
In order to produce cultured material in large quantities, it is desirable to use roller bottles. The use of roller bottles in tissue culture involves plating cells on the interior surface of a cylindrical container, providing the cells with an appropriate growth medium and rotating the container while the cells grow. The application of culturing techniques to roller bottle technology is necessary if a culture is to be mass produced. Roller bottles have large surface areas and permit easy exchange of the medium and gases. Because of the sensitivity and difficulty of culturing chondrocytes, roller bottle technology was heretofore considered unsuitable for the culturing of cartilage tissue. However, it has been found that good growth of cartilage tissue may be achieved in roller bottles from cells produced according to the above methods.

The cultures are maintained in Ham's F-12 medium supplemented with the antibiotic and antifungal agents described above as well as with 50 micrograms of ascorbic acid per ml. and 15% bovine serum. The cells are cultured at 37° in a humidified atmosphere containing at least 5% carbon dioxide. The medium is changed every other day. After about 10 days, nodules appear which contain extensive extracellular matrix chemically identified as being cartilaginous in nature.

AIF may be extracted from the chondrocyte cultures as well as the collected growth media according to the methods described in my Patent No. 4,042,457.

Example 1

About 10^8 chondrocytes are prepared and cultured by the above described method in a roller bottle having 500cm^2 of interior surface to achieve a



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plated density of about 2×10^5 cells per cm^2 of interior surface area. After 16 days the culture, which contains an estimated 3×10^8 cells has an extracellular matrix which morphologically and biochemically resembles the extracellular matrix of the adolescent bovine cartilage tissue from which the chondrocytes are obtained.

Example 2

2×10^6 chondrocytes are prepared and cultured by the above described method in a 35 mm culture plate (a density of plated cells of about 2×10^5 cells/ cm^2) for 16 days in the presence of ^3H -proline (10 micro Ci/ml). The scraped cells and medium combined are brought to 25% saturation with ammonium sulfate and the precipitate is collected by centrifugation following an overnight incubation at 4°C . The pellet is dissolved in 0.5 M acetic acid containing 1 mg/ml type I carrier collagen and 10 micrograms per ml. pepsin. Digestion is carried out for 10 hours at 4°C . The collagen mixture is brought to pH 8 and centrifuged to remove undigested material. The supernatant is again brought to 25% saturation with ammonium sulfate to precipitate the protein. The pellet produced by centrifugation is dissolved in 0.5 M acetic acid and exhaustively dialyzed against the same solution. The collagen, free of labeled unincorporated amino acid is lyophilized and subsequently dissolved in 6 M urea with 1% SDS.

The collagen is denatured at 70°C for 30 hours and electrophoresed under normal and reducing conditions according to Laemli, Nature, 227, 680, (1970).

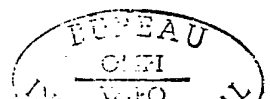
A 5% running gel separates the alpha 1 chains of type II collagen from the alpha 2 chains of type I collagen and the ratios of labeled alpha 1 to alpha 2 chains is determined. The band containing the alpha 2

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chains of the carrier type I collagen contains no label while the band containing alpha 1 chains is highly labeled indicating the production of only collagen type II by the culture.

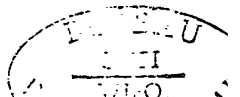
5 While the invention has been described according to the best mode presently known to the inventor, modifications obvious to one skilled in the art may be made without departing from the scope of the present invention which is limited only by the following
10 claims:



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CLAIMS:

1. A method for culturing chondrocytes from mammals to produce cartilage tissue comprising:
selecting cartilage tissue from adolescent mammals
providing at least about 60% viable chondrocytes,
5 preparing shavings from the cartilage tissue, digesting
said shavings to remove the extracellular matrix to
provide denuded cartilage cells, plating said denuded
cells in sufficient density to provide at least a
monolayer of cells so that said cells are in surface
10 contact with each other, and growing said cells in a
medium and obtaining therefrom a culture with an
extracellular matrix.
2. A method in accordance with Claim 1
wherein said cartilage shavings are obtained from the
15 mammalian articular cartilage.
3. A method for producing anti-invasion
factor comprising the steps of: selecting cartilage
tissue from adolescent mammals providing at least about
60% viable chondrocytes, preparing shavings from the
20 cartilage tissue, digesting said shavings to remove the
extracellular matrix to provide denuded cartilage cells,
plating said denuded cells in sufficient density to
provide at least a monolayer of cells so that said cells
are in surface contact with each other, growing said
25 cells in a medium and obtaining therefrom a culture with
an extracellular matrix, and extracting the
anti-invasion factor produced by said culture.
4. A method in accordance with Claim 3
wherein said cartilage shavings are obtained from the
30 mammalian articular cartilage.
5. A method for culturing chondrocytes to
produce cartilage tissue comprising: selecting mammalian



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cartilage tissue providing at least about 60% viable chondrocytes, preparing shavings from the cartilage tissue, digesting said shavings to remove the extracellular matrix to provide denuded cartilage cells, said shavings digested in separate mediums including; a
5 medium containing from about .5% to about 2% w/v pronase, a medium containing from about 0.1 to about 1% w/v collagenase, and a medium containing from about .1% to about 3% w/v of an enzyme selected from the group
10 consisting of trypsin and testicular hyaluronidase, plating said denuded cells in sufficient density to provide at least a monolayer of cells so that said cells are in surface contact with each other, and growing said cells in a medium and obtaining therefrom a culture with an extracellular matrix.

15 6. A method for producing anti-invasion factor comprising the steps of: selecting mammalian cartilage tissue providing at least about 60% viable chondrocytes, preparing shavings from the cartilage tissue, digesting said shavings to remove the
20 extracellular matrix to provide denuded cartilage cells, said shavings digested in separate mediums including; a medium containing from about .5% to about 2% w/v pronase, a medium containing from about 0.1 to about 1% w/v collagenase, and a medium containing from about .1%
25 to about 3% w/v of an enzyme selected from the group consisting of trypsin and testicular hyaluronidase, plating said denuded cells in sufficient density to provide at least a monolayer of cells so that said cells are in surface contact with each other, growing said
30 cells in a medium and obtaining therefrom a culture with an extracellular matrix, and extracting the anti-invasion factor produced by said culture.

7. A method in accordance with Claim 5 or Claim 6 wherein said shavings are digested in the

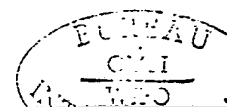
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presence of mammalian serum.

8. A method for culturing chondrocytes to produce cartilage tissue comprising: selecting mammalian cartilage tissue providing viable chondrocytes,
5 preparing shavings from the cartilage tissue, digesting said shavings to remove the extracellular matrix to provide denuded cartilage cells, plating said denuded cells in a cylindrical container in sufficient density to provide at least a monolayer of cells so that said
10 cells are in surface contact with each other, and providing a growth medium and rotating said container to grow said cells and produce a culture with an extracellular matrix.

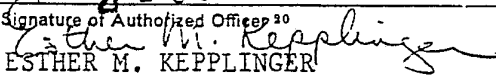
9. A method for producing anti-invasion factor
15 comprising the steps of: selecting mammalian cartilage tissue providing viable chondrocytes, preparing shavings from the cartilage tissue, digesting said shavings to remove the extracellular matrix to provide denuded cartilage cells, plating said denuded cells in a
20 cylindrical container in sufficient density to provide at least a monolayer of cells so that said cells are in surface contact with each other, providing a growth medium and rotating said container to grow said cells and produce a culture with an extracellular matrix, and
25 extracting the anti-invasion factor produced by said culture.



INTERNATIONAL SEARCH REPORT

International Application No

PCT/US81/00511

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ³				
According to International Patent Classification (IPC) or to both National Classification and IPC INT. CL. ³ C12N 5/00; C12P 21/00 U.S. CL. 435/68,240				
II. FIELDS SEARCHED				
Minimum Documentation Searched ⁴				
Classification System	Classification Symbols			
U.S.	435/68,240; 424/95,177			
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁵				
CHEMICAL ABSTRACTS, VOLUMES 76-92, 1972-1980				
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴				
Category ⁶	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸		
A	METHODS IN ENZYMOLOGY, VOLUME LVIII, CELL CULTURE, ISSUED 1979, WILLIAM B. JAKOBY ET AL, EDITORS, MARK M. BASHOR, "DISPERSION AND DISRUPTION OF TISSUES", MICHAEL KLAGSBRUN, "LARGE-SCALE PREPARATION OF CHONDROCYTES", PAGES 13,14,119-131,449,450,560-564, FOR CLAIMS 5-7, SEE PAGES 119-131.	1-9		
A	EXPERIMENTAL CELL RESEARCH, 108, ISSUED 1977, P.K. MÜLLER ET AL, "IMMUNO-CHEMICAL AND BIOCHEMICAL STUDY OF COLLAGEN SYNTHESIS BY CHONDROCYTES IN CULTURE", PAGES 47-55.	1-9		
A	US,E, RE-30,239, PUBLISHED 25 MARCH 1980, KUETTNER ET AL.	3,4,6,7,9		
A	FEBS LETTERS 37(1), ISSUED NOVEMBER 1973, OLE W. WIEBKIN ET AL, "THE INHIBITION OF SULPHATE INCORPORATION IN ISOLATED ADULT CHONDROCYTES BY HYALURONIC ACID", PAGES 42-46, SEE PAGE 42, LINES 4-7, UNDER 2.2 CHEMICALS.	7		
A	US,A, 3,450,598, PUBLISHED 17 JUNE 1969, WELSH ET AL.	8,9		
<p>¹⁵ * Special categories of cited documents:</p> <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none;"> <p>"A" document defining the general state of the art</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document cited for special reason other than those referred to in the other categories</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> </td> <td style="width: 50%; border: none;"> <p>"P" document published prior to the international filing date but on or after the priority date claimed</p> <p>"T" later document published on or 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</p> <p>"X" document of particular relevance</p> </td> </tr> </table>			<p>"A" document defining the general state of the art</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document cited for special reason other than those referred to in the other categories</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p>	<p>"P" document published prior to the international filing date but on or after the priority date claimed</p> <p>"T" later document published on or 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</p> <p>"X" document of particular relevance</p>
<p>"A" document defining the general state of the art</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document cited for special reason other than those referred to in the other categories</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p>	<p>"P" document published prior to the international filing date but on or after the priority date claimed</p> <p>"T" later document published on or 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</p> <p>"X" document of particular relevance</p>			
IV. CERTIFICATION				
Date of the Actual Completion of the International Search ²	Date of Mailing of this International Search Report ²			
14 JULY 1981	21 JUL 1981			
International Searching Authority ¹	Signature of Authorized Officer ²⁰			
ISA/US	 ESTHER M. KEPPLINGER			