In the Matter of

CERTAIN LIMITED-CHARGE CELL CULTURE MICROCARRIERS

Investigation No. 337-TA-129

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United States International Trade Commission / Washington, D.C. 20436

UNITED STATES INTERNATIONAL TRADE COMMISSION

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COMMISSION ACTION AND ORDER

Introduction

The United States International Trade Commission has concluded its investigation under section 337 of the Tariff Act of 1930 (19 U.S.C. § 1337) of alleged unfair methods of competition and unfair acts in the unlawful importation of certain limited-charge cell culture microcarriers into the United States, or in their sale, by the owner, importer, consignee, or agent of either, the alleged effect or tendency of which is to destroy or substantially injure an industry, efficiently and economically operated, in the United States, or to prevent the establishment of such an industry, or to restrain or monopolize trade and commerce in the United States.

The complainants are Flow General, Inc. and Flow Laboratories, Inc., of McLean, Virginia, and the Massachusetts Institute of Technology, of Cambridge, Massachusetts. The respondents named in the notice of investigation were AB

Fortia, Pharmacia AB, and Pharmacia Fine Chemicals AB, all of Uppsala, Sweden, and Pharmacia, Inc., of Piscataway, New Jersey.

This Action and Order provides for the final disposition of investigation No. 337-TA-129 by the Commission. It is based upon the Commission's determination, made in public session at the Commission meeting of October 28, 1983, that there is no violation of section 337.

Action

Upon review of the administrative law judge's (ALJ) initial determination, the Commission has considered (1) the submissions filed by the parties; (2) the transcript of the evidentiary hearing before the ALJ and the exhibits accepted into evidence; (3) the ALJ's initial determination; and (4) the arguments and presentations made at the Commission's public hearing on September 15, 1983. The Commission, on October 28, 1983, determined that with respect to all respondents in investigation No. 337-TA-129, there is no violation of section 337 of the Tariff Act of 1930 in the importation into and sale in the United States of certain limited-charge cell culture microcarriers. For those issues addressed in the ALJ's initial determination that the Commission chose not to review, the initial determination has become the decision of the Commission.

Order

Accordingly, it is hereby ORDERED THAT--

1. Investigation No. 337-TA-129 is terminated as to all issues and all respondents;

2. The Secretary shall serve this Action and Order and the opinion issued in connection therewith upon each party of record in this investigation and upon the U.S. Department of Health and Human Services, the U.S. Department of Justice, the Federal Trade Commission, and the U.S. Customs Service; and

3. The Secretary shall publish notice of this Action and Order in the Federal Register.

By order of the Commission.

Kenneth R. Mason
Secretary

Issued: November 18, 1983

VIEWS OF THE COMMISSION 1/

The following opinion reflects the Commission's determination on review of the initial determination (ID) of the administrative law judge (ALJ) in Certain Limited-Charge Cell Culture Microcarriers, Inv. No. 337-TA-129. 2/ The ALJ issued his ID on June 6, 1983, in which he determined that there was no violation of section 337 of the Tariff Act of 1930 3/ on the basis that: (1) the patents involved are invalid; and (2) respondents have not unfairly refused to sell sieved beads for making microcarriers. Patent infringement, unauthorized manufacture abroad in accordance with the process claims of a U.S. patent, and the refusal to sell sieved beads were the only alleged unfair practices remaining in the investigation at the time the ALJ issued his ID. The ALJ found all the other elements of a violation of section 337 to exist.

We agree with the ALJ that there is no violation of section 337. However, we have also determined to modify the ID, as discussed below.

PROCEDURAL HISTORY

On July 19, 1982, Flow General, Inc. (Flow General), Flow Laboratories, Inc. (Flow), and the Massachusetts Institute of Technology (MIT) filed a complaint with the Commission under section 337 of the Tariff Act of 1930. A

1/ The following abbreviations are used in this opinion:
ALJ = Administrative Law Judge; ID = ALJ's Initial Determination;
CX = complainants' exhibit; RX = respondents' exhibit;
TR = transcript of evidentiary hearing before ALJ;
CTR = transcript of Commission hearing on ALJ's initial determination on violation and also on remedy, public interest, and bonding;
CHB = complainants' prehearing brief for the Commission hearing;
RHB = respondents' prehearing brief for the Commission hearing;
CPB = complainants' posthearing brief for the Commission hearing;
RPB = respondents' posthearing brief for the Commission hearing.

2/ The Commission's review was pursuant to Rule 210.56(c), 19 CFR § 210.56(c).

3/ 19 U.S.C. § 1337.

supplement to the complaint was filed on August 3, 1982.

On the basis of that complaint, as supplemented, the Commission instituted this investigation on August 19, 1982. 4/ The notice of investigation was subsequently amended, so that as the investigation reached the ALJ for decision, the amended notice of investigation defined its scope as the determination of whether there is a violation of section 337 in the importation of certain limited-charge cell culture microcarriers into the United States, or in their sale, by reason of alleged:

> (1) refusal to sell sieved beads;
>
> (2) direct infringement of the claims of U.S. Letters Patent 4,189,534 (the '534 patent) and U.S. Letters Patent 4,293,654 (the '654 patent);
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> (3) contributory infringement and induced infringement of the claims of said patents; or
>
> (4) unauthorized manufacture abroad in accordance with the process claims of U.S. Letters Patent 4,293,654,

the effect or tendency of which is to destroy or substantially injure an industry, efficiently and economically operated, in the United States, or to prevent the establishment of such an industry, or to restrain or monopolize trade and commerce in the United States. 5/ 6/

4/ 47 Fed. Reg. 37312 (August 25, 1982).

5/ The amendment of the notice of investigation was accomplished by a joint motion of the parties, filed March 8, 1983, to amend the complaint and notice of investigation by withdrawing as alleged unfair methods of competition and unfair acts: (1) misappropriation of trade secrets; (2) false and deceptive advertising; and (3) false and disparaging comments about complainants. By Order No. 28, issued on March 14, 1983, the ALJ filed an initial determination pursuant to Rule 210.53(c), 19 CFR § 210.53 (c), granting that joint motion. On April 8, 1983, the Commission issued a notice that it would not review that initial determination. 48 Fed. Reg. 15966 (April 13, 1983).

6/ The complaint had alleged "misrepresentation to prevent issuance of patents." The Commission did not find it appropriate to include this count in its notice of investigation; the complainants concurred in the Commission's action. 47 Fed. Reg. 37312 (August 25, 1982); See, Memorandum of the Unfair Import Investigations Division to the Commission, UIID-F-241 (August 10, 1982).

The following four parties were named respondents in the notice of investigation: AB Fortia, Pharmacia AB, and Pharmacia Fine Chemicals AB, all of Uppsala, Sweden, and Pharmacia, Inc., of Piscataway, New Jersey.

After an evidentiary hearing, the ALJ issued the ID on June 6, 1983, finding that there is no violation of section 337. Both complainants and respondents petitioned for review of the ID. 7/

On July 14, 1983, the Commission issued a notice that it had determined to review the following portions of the ID: 8/

> 1. Validity of involved U.S. Letters Patent Nos. 4,189,534 and 4,293,654 (the patents) under 35 U.S.C. § 112, 35 U.S.C. § 102 (prior art Sephadex A-50 product only), and 35 U.S.C. § 103.
>
> 2. Infringement of the patents by respondents' CYTODEX 3 product.
>
> 3. Whether there is an "industry . . . in the United States", within the meaning of section 337.
>
> 4. Whether the importation or sale of respondents' CYTODEX products which are found to be involved with unfair practices have the effect or tendency to destroy or substantially injure such an industry.

On September 15, 1983, the Commission held a hearing on those portions of the ID it had determined to review and on relief, the public interest, and bonding.

PARTIES

Complainant MIT is a Massachusetts corporation and a research and educational institution having its principal place of business at 77

7/ Rule 210.54, 19 CFR § 210.54, governs petitions for review. That rule provides the following standards for granting such petitions:

> (A) A finding or conclusion of material fact [in the ID] is clearly erroneous;
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> (B) A legal conclusion is erroneous, without governing precedent, rule or law, or constitutes an abuse of discretion; or
>
> (C) The determination is one affecting Commission policy.

8/ 48 Fed. Reg. 32878 (July 19, 1983), as amended by 48 Fed. Reg. 36011 (August 8, 1983).

Massachusetts Avenue, Cambridge, Massachusetts 02137. MIT is the owner by assignment of the '534 patent and the '654 patent.

Complainant Flow General is a Delaware corporation having its principal place of business at 7655 Old Springhouse Road, Mclean, Virginia 22102. Flow General is engaged in the manufacture and sale of products for cell culturing, microtitration, and clinical diagnostic assays. Flow General is the exclusive licensee of MIT under the '534 and '654 patents.

Complainant Flow is a Maryland corporation and a wholly-owned subsidiary of Flow General. Its principal place of business is also 7655 Old Springhouse Road, McLean, Virginia 22102. Flow was established in 1961 to manufacture products for cell culturing, including media and sera required for cell growth.

Respondent AB Fortia is a Swedish corporation having its principal place of business at Uppsala, Sweden, (018) 163000. AB Fortia is the parent corporation of respondents Pharmacia Fine Chemicals AB and Pharmacia, Inc.

Respondent Pharmacia AB is a Swedish corporation having its principal place of business at Uppsala, Sweden, (018) 163000. Pharmacia AB is affiliated with respondents Pharmacia Fine Chemicals AB and Pharmacia, Inc.

Respondent Pharmacia Fine Chemicals AB (Pharmacia) is a Swedish corporation and a subsidiary of AB Fortia, having its principal place of business at Uppsala, Sweden, (018) 163000.

Respondent Pharmacia, Inc., is a New Jersey corporation and a subsidiary of AB Fortia, having its principal place of business at 800 Centennial Avenue, Piscataway, New Jersey 08854.

TECHNOLOGY INVOLVED 9/

This investigation involves complex subject matter; thus, the issues raised in this investigation may be more clearly understood by an overview of the technology involved. The involved technology is the large-scale culturing of mammalian cells. More particularly, it is the large-scale culturing of mammalian cells on microscopic beads called microcarriers.

Mammalian cells synthesize many proteins which have experimental, clinical, and perhaps commercial value. 10/ In many cases, the best or only source of these proteins is by culturing the mammalian cells known to produce them. 11/ Techniques for culturing mammalian cells on a small, laboratory, scale have been known for some time; the problem has been in moving from small-scale laboratory culturing to large-scale culturing. Culturing mammalian cells on a large scale is much more difficult than culturing bacteria, yeasts, or molds because of the fragile and complex nature of mammalian cells, which have stringent nutritional and environmental requirements. Among these environmental requirements is the requirement for a solid surface or substrate on which to grow; only a few unusual mammalian cells will grow in suspension. Thus, the majority of mammalian cells are "anchorage-dependent."

9/ A more detailed discussion of the technology involved may be found in Feder and Tolbert, "The Large-Scale Cultivation of Mammalian Cells," Scientific American (January 1983); CX-219.

10/ Among these proteins is interferon, which is known to inhibit viral infection.

11/ A typical mammalian cell culture begins with a mammalian tissue which is dissociated into individual cells or groups of cells to form a mixture of cells known as an inoculum. The inoculum is introduced into an appropriate liquid growth medium, which ordinarily includes serum to provide components not yet identified, but which have been shown to be essential for cell growth. The pH, temperature, oxygen and carbon dioxide levels and osmotic pressure of the medium must be carefully controlled.

A number of laboratory vessels are suitable as substrates. The most effective and widely used vessel is the roller bottle, a cylindrical vessel partially filled with medium and continuously rotated about its long horizontal axis. Cells attach themselves to the inner surface of the cylinder, and the slow rotation exposes them alternately to the liqiud medium and the air.

However, roller bottles cannot provide a sufficiently large surface area to volume ratio for practical large-scale culturing. Various means have been devised to increase the surface area to volume ratio, such as growing the cells on spongy polymers, on arrays of thin tubing or hollow fibers, on stacks of thin plates, or on microscopically small beads known as microcarriers.

The use of microcarriers as a substrate for culturing mammalian cells was developed in 1967 by Anton L. van Wezel, of the Dutch National Institute for Public Health. Dr. van Wezel used commercially available anion exchange resin beads marketed by Pharmacia under the trade name DEAE-Sephadex A-50 for his microcarriers. Various microcarriers and microcarrier techniques have since been developed. In general, the technique involves suspending the microcarriers in a nutrient medium. An inoculum of anchorage-dependent cells is introduced into the medium. The cells attach to the beads, grow, and multiply.

The use of microcarriers has the advantage of closely approximating suspension culture, but there are some problems. Collisions between beads can injure cells, and such collisions become more frequent with high bead density and the agitation characteristic of suspension culture. Cell growth over the large surface to volume area of the microcarrier beads may also result in rapid nutrient depletion and build-up of toxic waste products.

PATENTS INVOLVED 12/

1. The '534 patent

United States Patent No. 4,189,534, entitled "Cell Culture Microcarriers", was issued February 19, 1980, to David W. Levine, William G. Thilly, Daniel I. C. Wang, and Jason S. Wong. The patent was based on application Serial No. 842,696, filed October 17, 1977, which was a continuation-in-part of application Serial No. 740,993, filed November 11, 1976. The '534 patent is assigned to complainant MIT. The United States Government has rights under this patent pursuant to NSF Grant No. BMS 7405676A01 and NIEHS Grant No. T01 ES 00063.

The '534 patent contains 20 claims, of which claims 1, 4, 7, 10, 12, 13, 19, and 20 are considered representative claims. 13/ It claims a method for using the microcarriers claimed in the '654 patent as a substrate for growing anchorage-dependent cells and recovering cell by-products from such cells.

2. The '654 patent

United States Patent No. 4,293,654, entitled "Cell Culture Microcarriers", was issued on October 6, 1981, to David W. Levine, William G. Thilly, Daniel I. C. Wang, and Jason S. Wong. The patent was based on application Serial No. 54,319, filed July 2, 1979, as a division of application Serial No. 842,696 (now the '534 patent), filed October 17, 1977, which was a continuation-in-part of application Serial No. 740,993, filed November 11, 1976. The '654 patent is assigned to complainant MIT. The

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12/ The '534 patent (CX-1) and the '654 patent (CX-2) are reproduced in the Appendix.

13/ TR at 76 (Prehearing Conference).

United States Government has rights under this patent pursuant to NSF Grant No. BMS 7405676A01 and NIEHS Grant No. T01ES 00063.

The '654 patent contains 9 claims, of which claims 1, 2, 4, and 7 are considered to be representative claims. 14/ It claims "cell culture microcarriers" having a "charge capacity" of 0.1 to 4.5 milliequivalents (meq) per gram "of dry, untreated microcarriers." 15/ The microcarriers are composed of beads "formed from polymers containing pendant hydroxyl groups," to which "positively charged amino groups" have been attached to provide the "charge capacity." 16/ These beads are generally porous. The preferred bead is one of cross-linked dextran, and the preferred charge carrying group is diethylaminoethyl (DEAE). Thus, the preferred microcarrier is a porous, cross-linked dextran bead to which DEAE groups have been attached to provide the requisite "charge capacity." 17/ The '654 patent also claims a method for producing the cell culture microcarriers it claims.

3. Terminology of the patents

To understand the claims of both patents more clearly, it is necessary to discuss the meaning of the terminology used in the claims, particularly the terms "cell culture microcarriers," "charge capacity," and "dry, untreated microcarriers." 18/

The term "cell culture microcarriers" is defined in the specification of each patent as "small, discrete particles suitable for cell attachment and growth." 19/

14/ TR at 76 (Prehearing Conference).
15/ Claim 1.
16/ Col. 5, l. 59 - col, 6, l. 2.
17/ Claimed in claim 3.
18/ To the extent that there remains any dispute as to the meaning of these terms, this discussion constitutes the Commission's disposition thereof.
19/ '534 patent, col. 4, ll. 30-33; '654 patent, col. 4, ll. 32-35.

The term "charge capacity," also referred to in some of the claims as "exchange capacity," is terminology used to characterize ion exchangers. The reason for this is that the claimed invention is, at least in its preferred embodiment, said to be an improvement over a prior art material, DEAE-Sephadex A-50, a well-known ion exchange bead, made by respondents and used, among other things, as a microcarrier. 20/

The charge capacity of an ion exchanger is a quantitative measure of its ability to take up exchangeable counter ions. The capacity may be expressed as total capacity or available capacity. The total capacity is the amount of charged and potentially charged groups per gram of dry ion exchanger; it is essentially a measure of the number of charged groups dispersed throughout the matrix of the ion exchange bead. The available capacity is the actual capacity obtainable under specified experimental conditions. It is dependent on the accessibility of functional groups, concentration and ionic strength of the surrounding liquid medium, the nature of the counter ions, and the selectivity of the functional groups towards them. In the claims, charge capacity refers to total charge capacity.

20/ An ion exchanger is an insoluble material containing chemically bound charged groups and mobile counter ions. The counter ion may be reversibly exchanged with other ions of the same charge without any changes of the insoluble matrix. If the matrix carries positive groups the counter ions will be negative. Such an ion exchanger will exchange negative ions and is therefore termed an anion exchanger. In the same way, if the matrix carries negative groups the counter ions will be positive. Since the positive ions are exchangeable, the term cation exchanger is used. DEAE-Sephadex A-50 is an anion exchanger. The presence of charged groups is a fundamental property of an ion exchanger. The total number of groups and their accessibility determine the capacity. See, Sephadex Ion Exchangers, A Guide to Ion Exchange Chromatography, RX-12, admitted to be prior art. CHB 14. See also, CHB, App. B. (Glossary of Technical Terms).

The term "dry, untreated microcarriers" refers to the polymer beads prior to treatment to attach the positive charge-carrying groups. In the case of the preferred embodiment these would be cross-linked dextran beads such as commercially available Sephadex G-50 made by respondents.

PRODUCTS

The products involved in this investigation are limited-charge cell culture microcarriers, which generally refers to cell culture microcarriers having a charge capacity less than that of DEAE-Sephadex A-50.

Complainants' microcarriers are sold under the mark SUPERBEAD. They are prepared from an uncharged cross-linked dextran product, Sephadex G-50, manufactured by respondents, to which are attached positively charged DEAE groups; it is not disputed that SUPERBEAD microcarriers come within the claims of the '654 patent. All of complainants' SUPERBEAD microcarriers are manufactured in Scotland, by Flow Laboratories, Ltd., 21/ and are marked "Made in U.K." 22/

Respondents import and sell three microcarrier products as follows: CYTODEX 1, a cross-linked dextran bead having DEAE charge groups attached throughout the bead; (b) CYTODEX 2, a cross-linked dextran bead having N, N, N-trimethyl-2-hydroxyaminopropyl (THAP) charge groups attached only at the outer surface of the bead; and (c) CYTODEX 3, a dextran bead coated with denatured collagen.

21/ See p. 37 et seq., infra.
22/ TR at 1071.

PATENT VALIDITY 23/

Under 35 U.S.C. § 282, patents are presumed to be valid. The burden of proving invalidity is on respondents. The ALJ found that both patents are invalid for: (1) indefiniteness under 35 U.S.C. § 112, second paragraph; (2) failure to meet the description requirement under 35 U.S.C. § 112, first paragraph; (3) failure to meet the enablement requirement under 35 U.S.C. § 112, first paragraph; and (4) obviousness of the claimed inventions under 35 U.S.C. § 103. The ALJ also found the '654 patent invalid for anticipation of the claimed invention under 35 U.S.C. § 102. We will address each of these questions separately.

1. Indefiniteness (35 U.S.C. § 112, second paragraph) 24/

The claims define the exchange or charge capacity of the claimed microcarriers in terms of milliequivalents per gram of "dry, untreated microcarriers," i.e., in terms of the weight of the polymer beads used as the starting material for making the claimed microcarriers. The parties refer to this method of defining the charge capacity as the "MIT basis." Respondents measure the charge capacity of their CYTODEX microcarriers in terms of the weight of the final (treated) microcarrier product and allege that this is the conventional method for expressing charge capacity. The parties refer to respondents' method of defining the charge capacity as the "Pharamcia basis" or the "conventional basis." The ALJ found that the claims were indefinite

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23/ Except where indicated, the issues and arguments relate to all claims in issue in the '654 and '534 patents; however, the arguments have been largely framed in terms of claim 1 of the '654 patent. Complainants appear to let the validity of the '534 patent rise or fall with the '654 patent. See also, TR at 960.

24/ ID at 12-18, 98-109.

because of the absence of any disclosure in the patents which relates the "MIT basis" to the "conventional basis," making it difficult, if not impossible, for members of the public to determine whether their microcarriers infringe the claims or not. The ALJ noted that the parties now agree on a conversion formula 25/ relating the "MIT basis" to the "conventional basis," but found that there is uncertainty as to the MW factor in the formula, which calls for the molecular weight of the charge-carrying moiety in its charged state, i.e., as attached to the polymer bead to impart a positive charge. He based his finding on evidence of instances where certain of the inventors had used a molecular weight for the charge-carrying DEAE moiety of the preferred embodiment which did not include the molecular weight of the chloride counter ion associated with it or included an additional chloride counter ion.

We determine that the claims are not invalid for indefiniteness under 35 U.S.C. § 112, second paragraph. A patent claim is a definition of the patented invention. The statute, 35 U.S.C. § 112, second paragraph, requires the patent specification to conclude with claims "particularly pointing out and distinctly claiming" the invention. The shorthand for this requirement is "definiteness." It is a requirement that claims be free from ambiguity so that the public may determine with reasonable certainty whether or not they infringe the claims. If the scope of the subject matter embraced by a claim

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25/ The conversion formula is:

$$CC_{MIT} = \frac{CC_{Ph}}{1-[CC_{Ph}\ (MW)\ (0.001)]}$$

Wherein: CC_{MIT} = Charge capacity on MIT basis, meq/gm dry untreated microcarrier.

CC_{Ph} = Charge capacity on Pharmacia or conventional basis, meq/gm dry treated microcarrier.

MW = Molecular weight of charge-supplying group in its charged state, i.e., as attached to the microcarrier bead. RHB App.; CHB App. C.

is clear, and if the patentee has not otherwise indicated that he intends the claim to be of a different scope, then the claim is definite. 26/

There is no serious dispute that the subject matter embraced by the claims here is clear, and there is no indication that the scope of the claims is intended to be different. 27/ The dispute is whether, as a practical matter, members of the public may have difficulty determining whether or not they infringe these claims because they might have difficulty ascertaining the appropriate molecular weight to be used in the conversion formula. We do not feel that this is a question of definiteness at all. However, assuming it to be a question of definiteness, respondents have failed to carry their burden. In the first place, it is clear that manufacturers can determine whether or not they infringe the claims without the necessity for any conversion formula since they can determine charge capacity directly on the "MIT basis" by weighing out the dry polymer beads prior to treatment to attach the charge-carrying moiety. This method is described in Example 1 of both patents. While this method is limited to manufacturers, such as respondents, there is no reason why it should not be sufficient. The parties agree there is an entire class of claims, referred to as product-by-process claims, in which the claimed product is defined in terms of a process for making it. 28/ They also agree that such claims are not invalid for indefiniteness merely

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26/ In re Borkowski, 422 F.2d 904, 909; 164 USPQ 642, 645 (CCPA 1970).

27/ Respondents argue in their posthearing brief that "there has never been a satisfactory explanation of what 'per gram of untreated microcarrier' means." RPB (Answers to Commission Questions) at 17. However, as noted above, it is manifest from the patents that this term refers to the dry, polymer (e.g., dextran) beads before treatment to attach the charge-carrying moiety. See p. 10, supra.

28/ CTR at 80-82; CTR at 111-112 (as corrected).

because only a manufacturer can determine infringement. 29/ Respondents have cited no authority why the present claims should be treated differently from product-by-process claims. 30/

In the second place, it has not been established that the relevant segment of the public would not be able to convert from the "conventional basis" to the "MIT basis." There is no dispute that the conversion can be made, and the parties agree on what the conversion formula is. 31/ The only dispute has to do with the alleged uncertainty of the molecular weight (MW) of the charge-carrying moiety, which is a factor in the conversion formula. The evidence for this uncertainty, however, does not relate to an uncertainty of what the molecular weight of the charge-carrying moiety is, but whether the molecular weight factor in the formula should include the molecular weight of the counter ion associated with the charge-carrying moiety. The evidence showed the use of three different molecular weights for the DEAE moiety, the preferred charge-carrying moiety: 100, 135, and 172 (Approx.). These are appropriate molecular weights depending on whether the molecular weight of the chloride counter ion associated with the DEAE moiety is included as part of

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29/ CTR at 80-82; CTR at 112 (as corrected). See, In re Hughes, 496 F.2d 1216; 182 USPQ 106 (CCPA 1974).

30/ They appear to agree that the present claims should not be treated differently. CTR at 12. However, they argue in their posthearing brief that "it is unlikely that an ion exchange manufacturer would perform special tests during its manufacture for a small customer for the small amounts used in cell culture and attempt to determine whether its product is within the MIT definition of charge capacity." RPB (Answers to Commission Questions) at 15. The point, however, is not whether they would perform such a test, but whether such a test is available, which it is. Respondents also argue that even manufacturers would be hampered by certain so-called "error factors." RPB (Answers to Commission Questions) at 16. As discussed infra, p. 17, these "error factors" have nothing to do with the direct determination of charge capacity on the MIT basis as set out in Example 1 of the patents.

31/ See note 25, supra.

the molecular weight factor in the formula and whether there are one or two chloride counter ions associated with each attached DEAE moiety. The parties agree that the molecular weight of the chloride counter ion should be included, and that there is only one of these associated with each attached DEAE moiety. As the ALJ found, it is a simple matter to determine the appropriate molecular weight to use in the formula, once this question is resolved; one simply adds the appropriate atomic weights obtained from a Periodic Table of the Elements. The parties substantially agree on the molecular weight factor to be used for the preferred embodiment. Respondents have not established that it is not within the skill of persons in the art to determine the proper molecular weight for use in the conversion formula whenever it becomes necessary to do so. When the question became important, as it did in the context of this case, the proper conclusion was readily drawn.

2. Description requirement (35 U.S.C. § 112, first paragraph) 32/

The ALJ found that the description requirement is not met for three different reasons. First, the ALJ found that the patents contain no disclosure of how to determine whether microcarriers whose charge capacity is characterized on the "conventional basis" are within the claims of the patents, which specify charge capacity on the "MIT basis." This is the same ground on which the ALJ made his finding of indefiniteness. 33/

Second, the ALJ found that the disclosure of the patents is inadequate to permit those skilled in the art to determine whether microcarriers characterized on the "MIT basis" are within the scope of the claims. The ALJ

32/ ID at 12-18, 109-124.
33/ See p. 11, supra.

found this inadequacy to be due to the failure of the patent disclosures to take into account the following "errors":

(1) a 7% error attributable to moisture retained in the untreated beads even after conventional drying to constant weight;
(2) a 2% error attributable to loss of free dextran during treatment of the beads to attach the charge-carrying moiety;
(3) a 5% error attributable to degradation of the cross-linked dextran beads during treatment of the beads to attach the charge-carrying moiety;
(4) a 2% error attributable to error in determining the correct molecular weight of the attached charge-carrying moiety. 34/

Third, the ALJ found that the specification of each of the patents contains an "alternative definition" of the claimed invention which purports to define the charge capacity of the microcarriers in hydrated form on the "conventional basis." The ALJ found that when the charge capacity of DEAE-Sephadex A-50 was adjusted to account for the hydration, it came within this "alternative definition." Pointing to the fact that the "alternative definition" is different from the definition of the invention in the claims, the ALJ found this "compounds the confusion" he had already found to exist with respect to the conversion formula.

We find that the patents are not invalid for failure to meet the description requirement. The first paragraph of section 112 provides that the "specification shall contain a written description of the invention . . . ," meaning the claimed invention. Original claims constitute their own description. 35/ Thus, the requirement is important only when the claims have

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34/ Using these "error factors," the ALJ "corrected" the claimed charge capacity range in the patents from 0.1-4.5 meq/g of dry, untreated microcarriers to 0.12-5.36 meq/g, which he found resulted in anticipation of the claimed invention by the prior art DEAE-Sephadex A-50 microcarriers. See the discussion of novelty, infra, p. 20.

35/ In re Koller, 613 F.2d 819, 204 USPQ 702 (CCPA 1980).

been amended during prosecution of the application at the Patent and Trademark Office (PTO), being a requirement that the new definition of the invention in an amended claim be based on a description originally in the specification. The requirement assures that the newly defined invention is entitled to the original filing date of the application. 36/ The invention defined in the claims is clearly described in the specification. The specification as originally filed clearly conveys to those skilled in the art the information that the inventors have invented the specific subject matter of the claims. This is sufficient. 37/

The ALJ's first reason clearly does not relate to whether the claims are supported by the original specification and in any event proceeds from an incorrect premise, i.e., that it would not be possible to determine infringement of such claims. 38/

As to the second reason, it also does not relate to whether the claims are supported by the original specification and in any event proceeds from an incorrect premise, since the "errors" referred to by the ALJ have nothing to do with the direct determination of charge capacity on the "MIT basis" as set forth in Example 1 of the patents. The 7% error attributable to the retained moisture after drying to constant weight is in fact included in the weight of the dry, untreated microcarriers in Example 1, by definition of the word "dry". 39/ The 2% error attributable to washing away of free dextran and the 5% error attributable to degradation during treatment to attach the charge-carrying moiety obviously have nothing to do with the determination of

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36/ See, In re Smith, 481 F.2d 910; 178 USPQ 620 (CCPA 1973), and Chisum, Patents § 7.04.

37/ Smith, supra, 481 F.2d at 914; 178 USPQ at 624.

38/ See p. 13, supra.

39/ See, e.g., TR at 759-760.

the weight of the untreated beads and thus can have nothing to do with the direct determination of charge capacity on the "MIT basis." The 2% error attributable to the determination of the molecular weight of the charge-carrying moiety has nothing to do with the determination of the weight of the untreated dextran beads in Example 1 of the patents, because at that point the beads, being untreated, do not have the charge-carrying moiety attached to them. In addition, the evidence relied on to find that these errors exist was the testimony of an employee of respondents who, though an expert in his field, admitted he had not carried out any examples in the patents 40/ and whose testimony consisted essentially of estimates. Such testimony, in our view, should not be given great weight.

As to the third reason, the existence of an "alternative definition" does not detract from the fact that the claims are clearly supported by the original specification. In any event, there can be no confusing the description of the claimed invention with the "alternative definition," since the former clearly expresses charge capacity on the "MIT basis" and the latter clearly does not. 41/

3. Enablement requirement (35 U.S.C. § 112, first paragraph) 42/

The ALJ found that the specifications do not teach those of ordinary skill in the art how to use those claimed microcarriers having a charge

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40/ TR at 2561.

41/ The ALJ's finding that the prior art DEAE-Sephadex A-50 microcarrier has a charge capacity within the "alternative definition" has nothing to do with the description requirement. Rather, it relates to novelty. However, since the "alternative definition" is not the definition of the invention set forth in the claims, it is immaterial to the question of novelty of the claimed invention.

42/ ID at 12-18, 109-124.

capacity of less than 0.9 meq/g ("MIT basis"), i.e., that segment of the claimed range from 0.1 to 0.9 meq/g. The ALJ recognized that cell culturing is an art, but pointed to testimony by one of respondents' witnesses as demonstrating that those skilled in the art could not employ lower charge capacity microcarriers to effect good cell growth without first determining "by complex, drawn out experimentation which, if any, of these lowest charge microcarriers would effect good cell growth, and then only after testing each such material with a variety of anchorage-dependent cells, nutrient media, pH conditions, etc." 43/

The enablement requirement set out in the first paragraph of section 112 requires that the specification contain sufficient information to enable a person skilled in the relevant art to make and use the invention. We find that the claims are not invalid for failure of the specification to comply with the enablement requirement. 44/

It may be true that those of ordinary skill in the art may have to experiment with various culturing parameters before being able to use a microcarrier having a charge capacity of less than 0.9 meq/g. The patents specifically contemplate such experimentation and do not distinguish what experimentation might be required on the basis of charge capacity. Experimentation is not inconsistent with enablement, providing that it is not undue. 45/ Thus, the fact that experimentation may be complex, as testified to in this case, does not necessarily make it undue, if the art typically

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43/ ID at 119.

44/ We note that complainants are correct that the question of enablement does not arise with respect to claims 4, 10, and 19 of the '534 patent and claims dependent therefrom since these claims cover only microcarriers above 0.9 meq/g, about which there is no dispute as to enablement.

45/ In re Angstadt and Griffin, 537 F.2d 498; 190 USPQ 214 (CCPA 1976).

engages in such experimentation. This appears to be the case with cell culturing. 46/ 47/

4. Anticipation (35 U.S.C. § 102) 48/

The ALJ found that the claims of the '654 patent are anticipated by the prior art DEAE-Sephadex A-50 microcarrier, known to have a charge capacity of 3.5 ± 0.5 meq/g ("conventional basis"). The ALJ found that this indicates that a DEAE-Sephadex A-50 microcarrier existed with a charge capacity of 3.0 meq/g ("conventional basis") and found that when this charge capacity is converted using the agreed-upon conversion formula, a value of 5.01 meq/g ("MIT basis") is obtained, which he found to be within the "corrected" claimed range of 0.12 to 5.36 meq/g ("MIT basis"). 49/ The "corrected" range was computed by taking into account certain "error factors." These "error factors" are those referred to in the discussion of the description requirement. 50/

We find that the claims are not invalid for anticipation by DEAE-Sephadex A-50. Even if some DEAE-Sephadex A-50 beads were manufactured with a charge capacity of 3.0 meq/g ("conventional basis"), for the reasons discussed above,

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46/ We note that there is no dispute as to the operability of CYTODEX 2, which is admittedly within the claims of the patent and has a charge capacity of less than 0.9 meq/g. Respondents argue that the operability of CYTODEX 2 does not necessarily show enablement. This may be true, but it is respondents' burden to show nonenablement. This they have failed to do.

47/ The ALJ found that "the disclosure of the suit patents is inadequate to enable those skilled in the art to make or use microcarriers having charge groups concentrated only on their surfaces." ID at 117. However, this finding is not relevant to our analysis since the claims do not contain such a limitation.

48/ ID at 18-25, 124-147.

49/ The ALJ did not find the '534 patent claims anticipated. Respondents' argument with respect to the '534 patent is such that it depends on anticipation of the '654 patent claims. See also note 23, supra.

50/ See p. 16, supra.

this charge capacity must be converted and then compared with the actual charge capacity expressed in the claims as they appear in the patent, not as "corrected" by the ALJ. Such a comparison shows that DEAE-Sephadex A-50 lies outside the claims and so does not anticipate the claimed invention. 51/

5. Obviousness (35 U.S.C. § 103) 52/

Even if a claimed invention is not identically disclosed in the prior art, section 103 provides that it is not patentable "if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains."

The ALJ, considering all the prior art references together, concluded that the claimed inventions would have been obvious to a person of ordinary skill in the art at the time they were made. We agree with the ALJ that the claims are invalid for obviousness under 35 U.S.C. § 103, but it is necessary to amplify the reasoning leading to our conclusion.

As the ALJ noted, the appropriate analysis to measure the extent to which respondents have carried their burden with respect to obviousness is that set out by the Supreme Court in Graham v. John Deere Co., 383 U.S. 1, 17 (1966), wherein the Court stated:

51/ In a footnote in their posthearing brief, respondents perform a computation purporting to "correct" the converted charge capacity of DEAE-Sephadex A-50 and then compare it with the claims as set out in the patents. RPB (Answers to Commission Questions) at 8. This argument is inconsistent with the conversion formula agreed upon by the parties, which purports to be complete. It also comes late and depends on the (doubtful) existence of the so-called "error factors."

52/ ID at 25-38, 147-169.

> Under § 103, the scope and content of the prior art are to be determined; differences between the prior art and the claims at issue are to be ascertained; and the level of ordinary skill in the pertinent art resolved. Against this background, the obviousness or nonobviousness of the subject matter is determined.

Therefore, we must examine the scope and content of the prior art and ascertain the differences between the prior art and the claims. While numerous prior art references are of record and were considered by the ALJ, our discussion is limited to the most pertinent references.

A. DEAE-Sephadex A-50; Pharmacia brochure 53/

The use of DEAE Sephadex A-50 as a microcarrier is of fundamental importance in assessing the question of obviousness. It is mentioned in numerous references, and warrants an introductory discussion. DEAE-Sephadex A-50 is an ion exchange resin which had been manufactured by respondents for many years prior to the filing date of the patents. It is sold by respondents in the form of small, porous beads for use in ion exchange chromatography. These beads comprise an inert cross-linked dextran matrix to which DEAE groups have been attached. The attached DEAE groups are dispersed throughout the matrix; each attached DEAE group bears a positive charge and is thus the charged group for this ion exchanger. The counter ion for DEAE-Sephadex A-50, as sold, is negatively-charged chloride ion. It is this ion which is exchangeable with other negatively-charged ions, i.e., anions, Thus, DEAE-Sephadex A-50 is an anion exchanger. The total capacity of DEAE-Sephadex A-50 is 3.5 $\pm$ 0.5 meq/g ("conventional basis"). At the time the parent

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53/ This discussion of DEAE-Sephadex A-50 is based on the numerous discussions of it in the prior art references, including the Pharmacia brochure entitled: Sephadex Ion Exchangers, A Guide to Ion Exchange Chromatography (RX-12).

application for the patents was filed, it was known that ions in the vicinity of the charged groups competed for these groups and that the greater the ionic strength, the greater the competition for these binding sites. This binding of the charged groups results in a reduced capacity.

The claimed invention is essentially identical to DEAE-Sephadex A-50, except for having a lower total charge capacity.

B. Van Wezel Nature article 54/

In October 1967, A. L. van Wezel reported the successful use of DEAE-Sephadex A-50 beads as microcarriers for growing mammalian cells, including anchorage-dependent diploid and primary cells. Van Wezel indicated that cell adherence and growth appeared to be due to the positive charge on DEAE-Sephadex A-50, noting that this was not surprising in view of the fact that mammalian tissue cells are negatively charged. Van Wezel did not state any lower limit for positive charge beyond which cells would not attach and grow. Van Wezel reported that the maximum growth rate of the cells in his microcarrier cultures was about the same as in (conventional) monolayer cultures, but that higher cell concentrations could be achieved by changing the medium. Van Wezel closed his report by stating: "The optimal conditions for culturing cells and viruses by this culture method have still to be found."

C. Van Wezel et al. Biotechnology and Bioengineering article 55/

In 1969, van Wezel and his coworkers again reported the use of DEAE-Sephadex A-50 beads as a microcarrier for growing anchorage-dependent

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54/ A. L. van Wezel, "Growth of Cell-Strains and Primary Cells on Microcarriers in Homogeneous Culture," Nature 216:64 (1967); CX-5.

55/ Van Wezel et al., "Homogeneous Cultivation of Animal Cells for the Production of Virus and Virus Products," Biotechnology and Bioengineering, 11:875 (1969); RX-17.

mammalian cells. Among other things, they noted that when the quantity of DEAE-Sephadex A-50 exceeded 2 mg/ml, a "toxicity phenomenon" was encountered "unless the beads are pretreated with serum." Noting the expense of serum pretreatment, they mentioned that "[r]ecent experiments to obviate this expensive treatment by coating the beads with collodion show promise."

D. Van Wezel treatise article 56/

In 1973 van Wezel reported again on microcarrier cell culture in a treatise article. In that article van Wezel stated:

> From all materials tested DEAE-Sephadex A 50 appears to be most suitable to serve as microcarrier. The negatively charge tissue cells adhere very easily to the positively charged DEAE-Sephadex A 50, and the density of DEAE-Sephadex beads, after swelling, is about the same as the density of the culture medium. A disadvantage is that a slight inhibition of cell growth is found at concentrations exceeding 1 mg DEAE-Sephadex A 50 per milliliter culture medium. This can be obviated by coating the beads with a nitrocellulose product.

The coating procedure which is said to overcome the "slight inhibition of cell growth" at higher microcarrier concentrations is described in detail in the article. 57/

56/ Van Wezel, "Microcarrier Cultures of Animal Cells," Tissue Culture, Methods and Applications, P. F. Kruse and M. K. Patterson, eds., Academic Press, New York, pp. 372 ff. (1973).

57/ The article also notes that satisfactory results were obtained with DEAE-Sephadex A 25 and QAE-Sephadex ion exchange beads, which have charge capacities of 3.5 $\pm$ 0.5 and 3.0 $\pm$ 0.4 meq/g ("conventional basis"). Plastic beads, such as specially treated polystyrene and nylon II, were found to be unsatisfactory because cells did not adhere firmly enough to them. Van Wezel also reported the apparently successful use of Spherosil beads (a special kind of porous glass bead) as a microcarrier by the Institut Pasteur, but noted that these beads may be "too heavy to be kept in suspension at low stirring speeds, while at high speeds the cells may not easily attach to the beads."

E. Horng and McLimans article 58/

In 1975, Horng and McLimans published an article on microcarrier culture of calf anterior pituitary cells. The abstract of that article states in pertinent part:

> Calf anterior pituitary cells were . . . successfully cultivated in a microcarrier suspension culture system. DEAE-Sephadex was demonstrated to be a satisfactory type of microcarrier. The cells readily attached to the bead and, after a short lag period, they actively proliferated on the bead surface to yield growth of a predominately epithelial cell type. Under specific conditions the microcarrier supported primary cell growth up to levels of 2 X 10^6 cells per ml. High bead concentrations inhibited cell growth. The inhibition could be overcome by using proportionately higher cell inoculum so that a concentrated culture with 5 X 10^6 cells per ml. was achieved. The inhibitory effect of high bead concentration was found to be due to the absorption of serum protein and certain growth enhancing factors.

F. Horng dissertation 59/

In March 1975, shortly after the Horng and McLimans article was accepted for publication, Horng, in his doctoral dissertation, described, among other things, the use of DEAE-Sephadex A-50 as a microcarrier for growing calf anterior pituitary cells. It states, in pertinent part (pages 157-159), as follows:

> 5.2.4 Absorption character and the growth inhibition
> Van Wezel and his coworkers reported cellular growth inhibition at high bead concentrations. The mechanism of such inhibition has not been reported. Instead, nitrocellulose was later employed for coating the beads without, however, showing the effectiveness of the coating. It should be noted that the concentrations of the

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58/ Chi-Byi Horng and William McLimans, "Primary Suspension Culture of Calf Anterior Pituitary Cells on a Microcarrier Surface," Biotechnology and Bioengineering, Vol. XVII, pages 713-722 (1975) (accepted for publication December 20, 1974); CX-47.

59/ Chi-Byi Horng, Culture of Mammalian Cells on Microcarrier Surface, Dissertation Submitted to the Faculty of the Graduate School of the State University of New York at Buffalo (March 1975); RX-7.

beads employed after coating was lower than that routinely employed in this study. Under these conditions we found no significant inhibitory action.

The inhibitory action appeared to be caused by the absorption of serum protein as well as a growth enhancing activity present in post trypsinization fluid. Experimental evidences can be summarized as follows:

1. In petri dish cultures subjected to statistical analysis, the inhibitory reaction was found to be caused by the absorption of medium components rather than the release of toxic substances. A serum supplement added to the absorbed medium substantially reduced the inhibitory reaction.
2. In petri dish cultures used beads proved to be superior to fresh bead preparations. On the other hand, used medium proved inferior to fresh medium for culture growth. Since the possibility of the release of toxic substance was excluded, it was evident that the beads removed some components from the medium.
3. Absorption of serum protein was quantitated. This was correlated with the inhibitory reaction.
4. In spinner cultures, the serum supplement reduced the inhibitory reaction without, however, totally eliminating the reaction. This suggested that absorption of components other than serum protein from the culture fluid may be involved.
5. By treating high bead concentrations with spent medium, cell growth can be enhanced. The presence of a growth enhancing activity in spent medium occurred within the first week of the culture.
6. The release of the growth enhancing activity did not take place during the culture period. After cell dissociation the post trypsinization fluid can be separated from the dissociated cells. This fluid showed the presence of the growth enhancing activity. Neither the cell homogenate from an equivalent amount of minced tissue, nor the amount of serum employed for trypsin inactivation, displayed the same activity.
7. By treating the post trypsinization fluid with beads the activity can be depleted.
8. The inhibitory reaction at high bead concentration can be eliminated by a combined treatment of beads with the post trypsinization fluid and serum.

Thus, it is believed that the inhibitory reaction at high bead concentration has essentially been overcome.

G. United States Patent No. 4,036,693 (Levine patent)

This patent, entitled "Treatment of Cell Culture Microcarriers," was issued to David W. Levine, William G. Thilly, and Daniel I. C. Wang on July 19, 1977, based on an application filed February 2, 1976. 60/ It discloses and claims, among other things, a method of treating positively charged microcarriers, such as those produced by reacting polydextran beads with DEAE, by contacting them with macromolecular polyanions, such as carboxymethylcellulose, prior to and/or during use in cultures. Such treatment is said to overcome "deleterious effects previously observed in attempts to use these microcarriers in cell culture systems". 61/ Specifically, the prior art microcarriers referred to are DEAE-Sephadex A-50, and the "deleterious effects" are those "which prevent good cell growth," as indicated in "van Wezel's published data." 62/ The patent also states:

> While not wishing to be bound by the following theory, it is believed that the macromolecular polyanion is effective in improving cell growth because it competes with medium and cell produced nutrients for absorptive sites on the microcarrier surfaces where cells do not attach. 63/

It is important to note that no specific macromolecular ion is required. The rationale given in the Levine patent for large (macro) molecular weight is "to provide sufficient charges upon the polyanion so that it will remain bound to bead surfaces once it becomes attached." 64/ Other than that, the only requirement is that the anion be "nontoxic to growing mammalian cells." 65/ Some specific macromolecular polyanions which are said to be suitable include

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60/ CX-6.
61/ Abstract, ll. 8-10.
62/ Col. 2, l. 35 - col. 3, l. 3.
63/ Col. 4, ll. 6-11.
64/ Col. 4, ll. 45-50.
65/ Col. 4, ll. 57-59.

"negatively charged polysaccharides and proteins," particularly carboxymethylcellulose. 66/

H. The Canadian patent 67/

The Canadian patent, owned by respondent Pharmacia AB, describes an anion exchanger particularly useful for the chromatographic separation of large molecules. Example 1 describes such an anion exchanger comprising cross-linked dextran to which positive-charge-carrying dimethylaminoethyl (DMAE) groups are attached. It has an anion exchange capacity of 1.9 meq/g ("conventional basis"), which is 2.4 meq/g ("MIT basis"), and thus is within the claims of the patents here. 68/ Example 4 of the patent describes a cross-linked dextran bead with DEAE groups attached and having a charge capacity of 2.75 meq/g on the "conventional basis." The Canadian patent generally discloses how to make anion exchangers with charge capacities from 0.1 to 6.0 meq/g, as set out in its claim 3.

The anion exchangers of the Canadian patent are identical in all essential respects to the claimed invention, except that there is no evidence that anyone attempted to grow cells on them prior to the filing date of the patents here.

I. Whatman DE 52 beads 69/

This material, commercially available since 1973, is an ion-exchange material composed of positive-charge-carrying DEAE groups attached to beads comprising a cellulose matrix. It has a charge capacity of 1.0 meq/g

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66/ Col. 4, l. 63 - col. 5, l. 5.
67/ No. 651,507, issued October 30, 1962.
68/ See, e.g., TR at 400.
69/ Described in, among other places, three Whatman brochures: RX-270, RX-271, RX-272.

("conventional basis"). It is not disputed that when this value is converted to the "MIT basis," it falls within the claimed range. The Whatman ion exchanger beads are identical in all essential respects to the claimed invention, except that there is no evidence that anyone attempted to grow cells on them prior to the filing date of the patents here.

J. Servacel DEAE-32 beads; 70/ Inooka article

This material, available since at least 1970, is an ion exchange material composed of cellulose beads to which positive-charge-carrying DEAE groups are attached. It has a charge capacity of 1.0 meq/g ("conventional basis"), which, when converted, falls within the narrowest of the claimed ranges. The Servacel ion exchange beads are identical in all essential respects to the claimed invention, except that there is no evidence that anyone attempted to grow cells on them prior to the filing date of the patents here. However, in 1969, Dr. Shoshi Inooka reported attachment of MH129F (mammalian) cells to "DEAE-cellulose (Serva)." 71/

K. The Levine Birgmingham Paper 72/

In September 1975, Dr. Levine, D. I. C. Wang, and Dr. Thilly presented a paper entitled "Optimizing Parameters for Growth of Anchorage-Dependent Mammalian Cells in Microcarrier Culture" at the First International Cell Culture Congress at Birmingham, Alabama. Like Horng's, their experiments

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70/ These beads are described in, among other places, two brochures: RX-273, RX-274.

71/ Inooka, "The Adsorption of Suspended MH129F Cells to DEAE Sephadex Particles," 20 Tohoku Journal of Agricultural Research, No. 1 (March 1969); RX-8.

72/ Levine et al., "Optimizing Parameters for Growth of Anchorage-Dependent Mammalian Cells in Microcarrier Culture," First International Cell Culture Congress, September 21-25, 1975, Birmingham, Alabama; CX-8.

eliminated the presence of a soluble toxic factor released by the beads and concluded that the "toxic effect" observed by van Wezel was due to "significant uptake of nutrients by the beads." 73/ They stated further that:

> Our solution to the problem of nutrient leaching is to add to the medium a negatively charged non-nutritive component to compete with the positively charged exchange sites on the beads. Use of carboxymethycellulose (CMC), a polyanion, has given excellent results." 74/

This is, of course, no more than what the Levine patent discloses. The authors then state:

> A second approach to the problem of nullifying nutrient absorption is also being pursued. In this case, we are attempting to synthesize a carrier which is optimal for cell adhesion and growth. A survey of possible carrier material is shown in Table II. The survey experiments consisted of bringing together cells, medium and test materials in plates. Two criteria for successful spinner operation were applied: adhesion of cells to the carrier surface and cell spreading with accompanying overgrowth. Our results confirmed the desireability of a positively charged surface. 75/

As noted above, similar evaluations had been done by Horng and van Wezel with the same result. In the Birmingham paper, the authors go on to state:

> Therefore, we are in the process of establshing minimum workable charge densities for cell adhesion and growth and are attempting to place charged groups on both impervious bead supports (such as polyethylene and polystyrene) and on uncharged Sephadex G-50. In the case of our studies with modified Sephadex G-50, the key concepts of our efforts are to either concentrate all charges at the surface, leaving the center of the bead uncharged, or simply to reduce the total milliequivalents per gram of carrier, and thus reduce the total nutrient uptake. 76/ [Emphasis supplied.]

73/ Birmingham paper, CX-8, p. 15.
74/ Birmingham paper, CX-8, p. 16.
75/ Birmingham paper, CX-8, p. 18.
76/ Birmingham paper, CX-8, p. 18.

There is a dispute as to whether the Birmingham paper is prior art. The conference at which the paper was orally presented was attended by 50-500 cell culturists. Prior to the conference, a copy of the paper was given to the head of the conference. Afterward, copies were distributed on request. These copies were distributed, without any restrictions, 77/ to as many as six persons more than one year before the filing date of the involved patents.

We believe that the distribution of copies of the Birmingham paper without restriction makes that paper a "printed publication," within the meaning of 35 U.S.C. § 102(b). In Garrett Corp. v. United States, 422 F.2d 874, 164 USPQ 521 (Ct. Cl. 1970), as in this case, there was no question that the reference was "printed," only whether it was a "publication." The court there found that "while distribution to government agencies and personnel alone may not constitute publication, distribution to [6] commercial companies without restriction on use clearly does." 78/

Our determination is consistent with In re Wyer, 655 F.2d 221, 210 USPQ 790 (CCPA 1981), which views "printed publication" as a unitary concept, concerned with "probability of dissemination" and founded on the public policy of 35 U.S.C. § 102(b) to prevent withdrawal of subject matter already in the public's possession. Under In re Wyer, a document may be deemed a "printed publication" upon a satisfactory showing that it has been disseminated or otherwise made available to the extent that persons interested and of ordinary skill in the subject matter or art, exercising reasonable diligence, can

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77/ CTR at 43.
78/ Garrett, 422 F.2d at 878; 164 USPQ at 424.

locate it. Here between 50 and 500 persons interested and of ordinary skill in the subject matter or art were actually told of the existence of the paper and informed of its contents by the oral presentation. The document itself was actually disseminated, without restriction, to at least 6 persons, on their request, indicating not only unrestricted actual dissemination but also indicating that any person in the art who knew of the paper (and at least 50 to 500 did) could have a copy for the asking.

In any event, it appears that complainants have admitted that the Birmingham paper is prior art. During the prosecution of the '534 patent, the Birmingham paper was cited by MIT in a Supplemental Citation of Prior Art. At the time this Supplemental Citation of Prior Art was filed, MIT apparently felt that the paper could only be available as prior art as a "printed publication," but appears to have argued in the Supplemental Citation of Prior Art that it was not in fact a "printed publication." The Examiner disagreed. In his next Office Action, he rejected all the then pending claims under 35 U.S.C. § 103 over the Birmingham paper. 79/ The Examiner did not state the basis for using the Birmingham paper as a prior art reference, nor did he respond to MIT's "printed publication" argument. We do not know whether the Examiner felt the Birmingham paper was available on another ground or whether he simply rejected MIT"s "printed publication" argument. In its next response, MIT argued only the merits of the Birmingham paper and did not contend that it was not available as prior art. 80/ A Notice of Allowance then issued.

79/ '534 Patent File History, CX-12, p. 363.
80/ '534 Patent File History, CX-12, p. 372.

During the subsequent prosecution of the '654 patent, some claims were allowed in the Examiner's first action and others were rejected under 35 U.S.C. § 112. No art rejections were made, but in the accompanying Notice of References Cited, the Examiner cited the Birmingham paper, thus indicating that it was available as a prior art reference. 81/ In its response, MIT did not take issue with this, and a Notice of Allowance was duly issued.

Thus, aside from the question of whether the Birmingham paper is a "printed publication," complainants have acquiesced in its treatment as prior art, effectively admitting it to be so. 82/

The ALJ found that the subject matter of the claimed invention involved two arts, ion exchange chemistry or organic synthesis and cell biology. He also found the level of skill to be high, requiring at least an undergraduate degree in chemistry and/or biology and two or three years of actual experience. 83/ These findings are not disputed here.

Viewing the foregoing, and the record as a whole, we conclude that the claimed inventions would have been obvious. The prior art DEAE-Sephadex A-50 ion exchange beads were well known to be useful as cell culture microcarriers. The only difference between DEAE-Sephadex A-50 beads and the claimed microcarriers is the lower charge capacity of the latter. The "toxicity" phenomenon noted by van Wezel with respect to high concentrations of DEAE-Sephadex A-50 beads was known to have been overcome by pretreatment with serum or a polyanion, such as nitrocellulose or carboxymethylcellulose.

81/ '654 Patent File History, CX-12, p. 49.
82/ In re Nomiya, 509 F.2d 566; 184 USPQ 607 (CCPA 1975).
83/ID at 154.

The Birmingham paper expressly states that to reduce the total charge capacity of DEAE-Sephadex A-50 would have the same effect as these pretreatments. 84/ 85/ The Canadian patent and the Whatman and Servacel products clearly show how to achieve this reduced charge capacity, and indeed indicate that anion exchange beads with such a reduced charge capacity were already commercially available. The claimed inventions would thus have been obvious.

PATENT INFRINGEMENT 86/

Complainants have alleged infringement by three of respondents' products: CYTODEX 1, CYTODEX 2, and CYTODEX 3. The ALJ found that all three of the products infringed the claims of the patents. Respondents did not petition for review of the ALJ's finding that CYTODEX 1 and CYTODEX 2

84/ The Birmingham paper is not necessary to demonstrate obviousness. The Levine patent expressly states that its polyanions bind to positively-charged groups on DEAE-Sephadex A-50 microcarriers. This binding of charged groups reduces the number of positively-charged groups available for binding cells. The same reduction may be achieved by reducing the number of charge groups, i.e., reducing the total charge capacity. See, e.g., Sephadex Ion Exchangers, A Guide to Ion Exchange Chromatography, RX-12.

Further, it is only necessary to have known of the association of fewer charge groups with overcoming the "toxicity" problem, no matter what the cause of that problem. Thus, it is not necessary to know whether the "toxicity" phenomenon is associated with competition for the position-charge-carrying sites by (negatively-charged) components of the nutrient media, thus depleting the medium of nutrients and making these nutrients unavailable for cell growth. There is considerable evidence that, at least at the time the claimed invention was made, this association was thought to exist, but complainants deny that there is now an acceptable theory sufficiently explaining the "toxicity" phenomenon, referring to testimony by Dr. Thilly in this case that he is unaware of such a theory. We note that Dr. Thilly and the other inventors offered this very theory in the Levine patent and the Birmingham paper.

85/ Complainants have argued that the Birmingham paper does not suggest any particular charge capacity range. See, e.g., CTR at 12. However, this is of little significance, since complainants' claimed range encompasses virtually the entire possible range below that of prior art DEAE-Sephadex A-50.

86/ ID at 44-49, 202-215.

infringe, but did petition for review of his finding that CYTODEX 3 infringes. Our review focuses on CYTODEX 3.

The ALJ found that , not collagen, is the charge-carrying moiety on CYTODEX 3 whose molecular weight must be included in the conversion formula. He then found that when the molecular weight of only the is used in the conversion formula, infringement is made out.

We find that complainants have not established that CYTODEX 3 infringes the claims. The function of any conversion formula is to account for the weight of all the moieties added to the dextran bead so that charge capacity in terms of the weight of the dextran bead alone can be ascertained. It is clear that collagen is attached to the dextran bead through the . 87/ Therefore, the collagen and must both be acounted for. The exclusion of collagen would mean that the conversion formula could not possibly account for the weight of the collagen. 88/

INDUSTRY IN THE UNITED STATES 89/

In order for the Commission to find a violation of section 337, there must exist an "industry, efficiently and economically operated, in the United

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87/ See, e.g., the representation at CHB-54, which complainants agree is "probably basically accurate and certainly the best representation that exists." CTR at 40.

88/ Respondents argue that even if CYTODEX 3 literally comes within the language of the claims of the patents, it does not infringe because the reverse doctrine of equivalents applies. This doctrine, set forth in Westinghouse v. Boyden, 170 U.S. 537 (1898), holds that even if a product comes within the language of a claim, it does not infringe if its mode of operation is totally different from that of the patented invention. We do not reach this issue.

89/ ID at 54-72, 224-252.

States." 90/ Relying on Certain Apparatus for the Continuous Production of Copper Rod, Inv. No. 337-TA-52, USITC Pub. No. 1017 (1979) (Copper Rod), the ALJ found that the two patents at issue in the subject investigation are "integrally related" and consequently concluded that the industry should be defined as a single industry encompassing operations under both patents. After analyzing the nature and the significance of complainants' operations in the United States, the ALJ concluded that these operations were sufficient to warrant a finding that they constitute an "industry . . . in the United States."

Even though we have disposed of the patent validity issue negatively, we continue to reach each of the elements of violation which are on review in this investigation. The Commission determined to review the question of industry because of its important policy implications. 91/

We determine that there are two industries, one encompassing complainants' operations under the '654 patent, and the other encompassing complainants' operations under the '534 patent. 92/ However, we also determine that the nature and significance of complainants' operations in the United States under the '654 patent do not justify treatment as an "industry . . . in the United States," but that complainants' operations under the '534 patent in the United States do justify treatment as an industry . . . in the United States."

90/ The question of the efficient and economic operation of a domestic industry has not been reviewed.

91/ See Rule 210.54(a)(ii), 19 CFR § 210.54(a)(ii).

92/ We note that the fact that the '654 patent is a division of the '534 patent means the two patents are directed to independent and distinct inventions as provided by 35 U.S.C. § 121, under which divisions may be required.

The '654 patent covers microcarriers; the '534 patent covers a method for obtaining cell by-products. Complainants use the '534 method to produce interferon. Combining the operations under these two patents is not justified because exploitation of the patents results in two clearly separate articles of commerce, i.e., microcarriers and interferon. In contrast to the present investigation, the Commission, in Copper Rod, found a single industry where apparatus and method patents and several trade secrets were involved. This finding was based on the fact that these property rights, as actually exploited, did not result in segregable products, but rather a single, integral system, "sold as a 'package' comprising apparatus components, licensing of patent and trade secret know-how, engineering and start-up operations, and other technical assistance, etc." 93/ Further evidence that the exploitation of the patents in the present investigation results in two distinct articles of commerce is provided by the products' identifiable, individual performances in the marketplace. The fact that the '654 microcarriers are used in the '534 process by complainants simply means that complainants' operations under the '534 patent constitute an internal, captive market for complainants' imported SUPERBEAD microcarriers covered by the '654 patent. In other words, combining operations under these two patents would be to confuse the market served by an industry with the industry itself.

1. Operations under the '534 patent

Complainant Flow has contracted to supply human fibroblast interferon to the National Cancer Institute. This operation, know as the Beta Interferon

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93/ Copper rod, supra, at 55.

Program, is conducted in the United States and employs the method of the '534 patent. 94/ Thus, there is an "industry . . . in the United States" under the '534 patent.

2. Operations under the '654 patent

As to the industry encompassing complainants' operations covered by the '654 patent (the patented microcarrier industry), the nature and significance of complainants' activities in the United States do not justify treatment as an industry "in the United States."

As the ALJ noted, complainants' SUPERBEAD microcarriers are manufactured entirely in Scotland. 95/ Indeed, they are marked "Made in the U.K.". Furthermore, complainants' SUPERBEAD microcarriers are packaged entirely in Scotland. In this investigation, the package insert which is printed in the United States is of little consequence. The ALJ divided the quality control tests for the SUPERBEAD microcarriers into four categories: (1) packaging check; (2) sterility; (3) physical chemistry; and (4) functionality. Initially, all quality control tests were performed in the United States. However, as Flow Labs Scotland's personnel became more adept at the techniques, certain quality control tests were performed on site in Scotland. Consequently, quality control tests have been performed in Scotland since at least 1981, and the tests that are performed in the United States are essentially redundant.

In his analysis, the ALJ focused particularly on the functionality test, noting that it was the single most important quality control test. From 1977 through June 1981, the functionality test was only performed by Flow in the

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94/ ID at 62.
95/ ID at 229.

United States. However, we note that in June 1981, Flow Labs began to perform the full functionality test in Scotland. It was not until 1982

functionality test was performed a second time in the United States on some shipments of SUPERBEAD microcarriers. 96/ Furthermore, the 17 percent value alleged to be added by this redundant test reflects intracompany pricing. The inclusion of the value of this test is also questionable since it appears to reflect the cost of the additional functionality testing for those SUPERBEADs used in the Beta Interferon Program

.97/ 98/ This additional functionality testing would not be relevant to the domestic industry defined by the '654 patent.

The ALJ found that by royalty payments and other means Flow supports research at MIT "in microcarrier technology." Flow conducts research and development which include "the Beta Interferon Program; production devoted to improving Superbead production protocols, and cell systems research devoted to studying and improving microcarriers, culture media, and other cell culturing factors." 99/ The ALJ found that between 1980 and 1982 Flow Labs U.S. spent on "Superbead development" and that for 1983, had been allocated to research and development, million to the Beta Interferon Program, and to cell research

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97/ At least of the total cost of complainants' SUPERBEAD microcarriers is the cost of the dextran beads. CTR at 15. These dextran beads are manufactured abroad by respondents as their Sephadex G-50 product. CTR at 35. Whether purchases originate in the United States or not is not relevant to an assessment of the significance of complainants' operations in the United States.

98/ The ALJ noted that of the kg of SUPERBEADs produced from 1977 to 1982, kg have been shipped to the United States, of which kg have been used in Flow's Beta Interferon Program. ID at 233.

99/ ID at 234.

and development. 100/ It is apparent to us that the vast bulk of the research and development relied upon relates only to the the Beta Interferon Program, i.e., operations under the '534 patent. Thus, we find that they cannot be considered as operations under the '654 patent. Furthermore, the extent to which the remaining research deals with operations under the '654 patent is not clear. 101/

The ALJ noted that Flow has a marketing network of regional sales representatives, but noted that they are responsible for "a full line of tissue culture products, so only a small percentage of their time is spent promoting or processing sale of Superbeads." 102/ We find the activities here to be no more than would be undertaken by any importer, and in any event they are of a minimal nature. 103/

Finally, the ALJ pointed to the technical product support provided to customers, analogizing it to the service activities in Certain Airtight Cast Iron Stoves, Inv. No. 337-TA-69, USITC Pub. No. 1126 (January 1981). 104/ We determine that complainants' "technical service" amounts only to product support and is not of the same nature as the repair and installation

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100/ ID at 241.

101/ The ALJ found that comparing the in sales of SUPERBEADs between 1978 and 1982 to the research and development expenditures "on Superbeads alone" between 1980 and 1982, complainants' "domestic activities related to quality assurance and development add relatively more to the product than is added abroad." ID at 245. However, this comparison overlooks

102/ ID at 234.

103/ See also Certain Miniature, Battery-Operated, All Terrain, Wheeled Vehicles, Inv. No. 337-TA-122, USITC Pub. No. 1300 (October 1982), aff'd, Schaper Manufacturing Co. v. U.S. International Trade Commission, -F.2d-(CAFC 1983).

104/ ID at 239.

activities found in Stoves; in any event, the significance of such activities is minimal.

Based on the record, we determine that the nature and extent of complainants' operations in the United States under the '654 patent are insufficient to constitute an "industry . . . in the United States."

INJURY

Even though we have disposed of the questions of unfair practices and industry, we continue to reach this last element of violation which is on review in this investigation. The injury issue in this case is whether the alleged unfair practices in the importation and sale of respondents' products have the effect or tendency to substantially injure the industry defined above. 105/ It is complainants' burden to establish such substantial injury and that such injury is caused by respondents' unfair practices.

To prevail under section 337, complainants must prove not only that respondents committed the unfair practices alleged, but also that respondents' unfair practices have the effect or tendency to substantially injure a domestic industry. Commission practice has emphasized the separate nature of the injury and unfair practice requirements; each element requires independent proof. The establishment of patent infringement does not release complainants from the burden of establishing substantial injury, or of showing the requisite causal connection between the imports and the injury. 106/

105/ The question of whether respondents' alleged unfair practices have prevented the establishment of an industry in the United States was decided adversely to complainants by the ALJ in his ID and is not on review.

106/ Chairman Eckes and Commissioners Stern and Lodwick reference the Recommended Determination of the ALJ in Certain CT Scanner and Gamma Camera Medical Diagnostic Imaging Apparatus, Inv. No. 337-TA-123 (March 4, 1983) at 180.

1. Operations under the '534 patent

These operations are the only operations which constitute an "industry . . . in the United States." However, the product of this industry is beta interferon, which respondents do not import. Therefore, there can be no injury to this industry within the meaning of section 337. 107/

2. Operations under the '654 patent

The ALJ found that there were two markets for microcarriers: "the traditional research market, consisting of laboratories and university research departments conducting experiments in cell culturing and requiring only small quantities of microcarriers, and the macro, or industrial market, consisting of vaccine and veterinary product manufacturers which engage in large-scale propagation of cells and cell by-products." 108/ He also found that "the greatest potential for large-volume sales of microcarriers is the industrial market"; indeed, he found that "[T]he future viability of the microcarrier technique appears to depend on its acceptance and widespread use by the industrial market." 109/ However, he found that "no potential large-scale user has adopted the microcarrier technique." 110/

Nevertheless, the ALJ found that "the failure of the industrial segment of the market to expand according to the parties' original expectations should not be allowed to obscure the presence of the laboratory market," and

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107/ Assuming that complainants' activities under both patents were combined to find an industry, our conclusion that there is no injury would be the same since the only operation "in the United States" under the '654 patent which could be included in such a combination is -

. . . This operation, to the extent it exists in the United States, is so minimal that the effective result of combining it with complainants' operations under the '534 patent would be to define the industry in terms of operations covered by the '534 patent alone. See p. 37, supra.

108/ ID at 254.

109/ ID AT 255.

110/ ID at 255.

then proceeded to conduct his injury analysis with respect to the laboratory market. 111/ He found the industry to have been injured in this market, and concluded that such injury was "substantial." He also found a tendency to substantially injure.

We find that complainants are not suffering substantial injury as a result of respondents' alleged unfair practices. We also find that there is no tendency to substantially injure the domestic industry that we have assumed to exist for purposes of this analysis. In assessing injury, it is important to note that only a proportion of Flow's SUPERBEAD microcarriers are sold to third parties; the are consumed by Flow itself to produce beta interferon under its contract for NCI. Flow's production capacity for SUPERBEAD microcarriers in 1982 was kg. 112/ kg of SUPERBEADs was produced in Scotland from 1977 to 1982. 113/ Of this, kg have been shipped to the United States, of which kg was for use in the Beta Interferon Program. The remaining kg appears to be completely accounted for by sales to third parties in the United States.

The industrial segment of the market, the only segment which might have been expected to make large-scale purchases, has, as the ALJ found, simply failed to adopt microcarrier technology. The remaining segment of the market, the "laboratory segment,"

However, even conducting an injury analysis on the basis of this laboratory

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111/ ID at 257-258.
112/ ID at 78.
113/ Production of the SUPERBEAD microcarriers in Scotland . ID at 78.

market, complainants have failed to show any substantial injury or tendency to substantially injure caused by the subject practices.

The "laboratory segment" comprises researchers in commercial establishments who are evaluating the microcarriers for potential commercial uses, nonprofit institutions, and Government laboratories. 114/ Any decline in sales to researchers in commercial establishments is properly attributable to nonacceptance of microcarriers for industrial use. As to Government laboratories, there can be no injury as a matter of law because the Government has a royalty-free license under both patents for any Government purpose, including the right to second source from anyone, including Pharmacia. 115/ This extends to Government-funded research in non-Government laboratories, which include some of the nonprofit institutions. A large proportion of such research was said to be Government funded. 116/ 117/

Thus, only a small portion of the laboratory market can possibly be injured as a result of respondents' activities. And even in this small portion, complainants have not met their burden. This is shown by evidence that in addition to the failure of the industrial market to accept microcarrier technology,

CYTODEX 1, which was introduced in 1978, the same year as complainants' SUPERBEAD, experienced

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114/ CTR at 63-65.
115/ CTR at 176, 179-183, 184.
116/ CTR at 185.
117/ Furthermore, we cannot overlook complainants' statement at the Commission hearing that there was no intent by MIT or Flow to enforce the patents against personnel in research laboratories, whether those research laboratories are nonprofit, other educational institutions, or commercial concerns. CTR at 176-177.

CYTODEX 2 was introduced in 1981, the year CYTODEX 1 and SUPERBEADs kg was sold in 1981, … for CYTODEX 1 and SUPERBEADs (approximately kg), although close to the for SUPERBEADs alone. On the other hand, CYTODEX 2 sales and the combined sales of CYTODEX 1 and CYTODEX 2

The ALJ found that between 1978 and 1982 respondents imported and sold kg of CYTODEX products, while Flow sold kg and used an additional kg of SUPERBEADs in its Beta Interferon Program. He concluded from this that "Pharmacia's market penetration is greater than percent, and their volume of imports, far from being de minimis, is substantial." 118/

While the ALJ noted that it was difficult to evaluate complainants' evidence of lost sales, he found some evidence that : (1) many of Flow's customers have purchased microcarriers from both Flow and Pharmacia, (2) many appear to have switched to Pharmacia, and (3) some customers have stopped buying microcarriers altogether. 119/ However, complainants' National Sales Manager was unable to confirm conclusively at trial Flow's allegedly lost sales to Pharmacia, but merely referred to purchases of respondents' products by customers of complainants. 120/ These may not necessarily be lost sales. The ALJ found that "Complainants' loss of customers appears to be the result of many factors, including a recessionary economy, initial difficulties with quality control, and customer difficulties with the technique," but concluded

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118/ ID at 262–263.
119/ ID at 260.
120/ ID at 260.

that "as a result of Pharmacia's market penetration, Flow has demonstrated loss of customers to Pharmacia." 121/

There is no clear evidence of lost customers. There is substantial evidence tht the subject imports were not bought as substitutes for complainants' products, 122/ because the record shows that many customers purchased from both complainants and respondents. Researchers tend to seek multiple sources of supply. Furthermore, any lost sales which might have occurred may be noninjurious sales under the Government's rights. Clearly some customers were lost for reasons having nothing to do with respondents. While market penetration may mean respondents have customers, market penetration alone does not mean that respondents obtained those customers at the expense of complainants. 123/ We find that under the facts of this investigation, the market penetration of respondents' microcarriers does not indicate substantial injury or tendency to injure.

The ALJ found that

124/

121/ ID at 262.
122/ Four other, noninfringing, microcarriers are available, two of which are sold at less than half the price of respondents' products.
123/ ID at 262.
124/ ID at 264.

While respondents' CYTODEX products are sold at a lower price than SUPERBEADs, underselling alone does not establish lost sales. In fact, there is an indication that lost sales are not occurring, i.e., customers are buying both products. Furthermore, because of the nature of the laboratory market, price does not appear to be an important factor.

The ALJ found that Flow had excess capacity to produce microcarriers, but stated that the main source of this excess capacity has been "the failure of microcarrier technology to achieve its anticipated acceptance in the industrial market." 125/ Nevertheless, the ALJ found that respondents' "significant market penetration, volume of sales and lower priced product is a contributing factor to Flow's excess capacity." We find that complainants' excess capacity is due to lack of acceptance of microcarriers in the potentially large industrial market, not the small laboratory market. The contributing factors discussed by the ALJ relate to the small laboratory market and, thus, any contribution they may have made is correspondingly small.

125/ ID at 268.

APPENDIX

United States Patent [19]
Levine et al.

[11] 4,189,534
[45] Feb. 19, 1980

[54] CELL CULTURE MICROCARRIERS

[75] Inventors: David W. Levine, Somerville; William G. Thilly, Cambridge; Daniel I. C. Wang, Belmont, all of Mass.; Jason S. Wong, Toledo, Ohio

[73] Assignee: Massachusetts Institute of Technology, Cambridge, Mass.

[21] Appl. No.: 842,696

[22] Filed: Oct. 17, 1977

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 740,993, Nov. 11, 1976, abandoned.

[51] Int. Cl.² C12B 3/00; C12K 9/00
[52] U.S. Cl. 435/2
[58] Field of Search 195/1.8

[56] References Cited

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3,904,480	9/1975	Hall et al.	195/1.7
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4,036,693	7/1977	Levine et al.	195/1.8

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651507 10/1962 Canada .

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Horng—Primary Culture of Mammalian Cells on Microcarrier Surface, PHD Thesis, State University of N.Y., Buffalo 1975.
Levine et al.—Proceedings from 1st International Cell Culture Congress (Sep. 21-25, 1975) Birmingham, Alabama.

Primary Examiner—Sam Rosen
Attorney, Agent, or Firm—Arthur A. Smith, Jr.; David E. Brook

[57] ABSTRACT

Improved cell culture microcarriers, and methods for their production and use, are disclosed herein. These improved microcarriers have positive charge capacities adjusted and/or controlled within a range suitable for good cell growth. One method for producing such improved microcarriers is by treating beads formed from polymers containing pendant hydroxy groups, such as dextran beads, with an aqueous solution of an alkaline material and a chloro- or bromo-substituted tertiary amine under precisely controlled conditions to produce the desired exchange capacity. The resultant positively charged microcarriers have been used in microcarrier cultures to produce outstanding growth of anchorage-dependent cells. Such cells can be harvested, or used for the production of viruses, vaccines, hormones, interferon or other cellular growth by-products.

20 Claims, 2 Drawing Figures

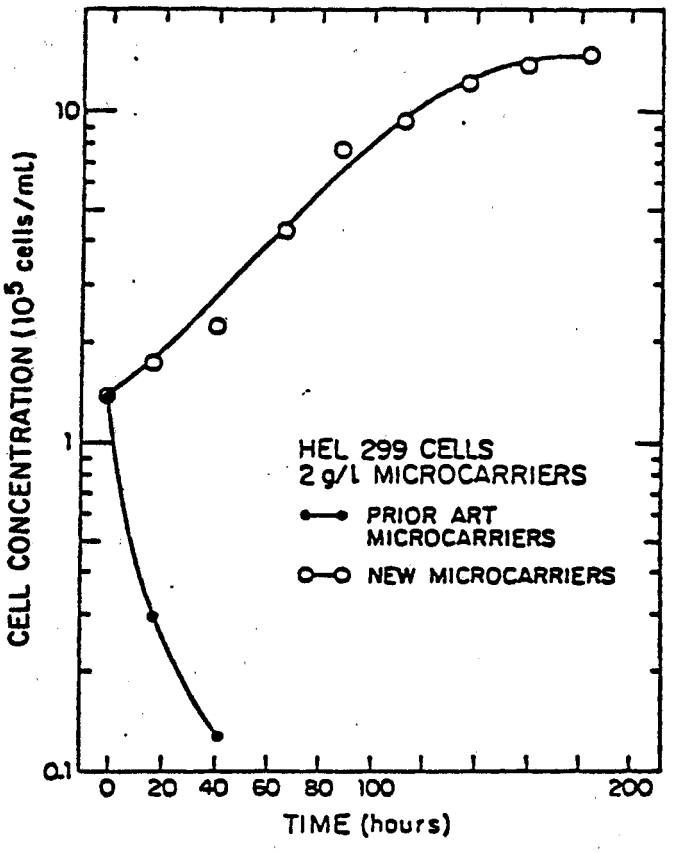

CELL CONCENTRATION (10^5 cells/mL)
10
1
0.1
0
20
40
60
80
100
200
TIME (hours)
HEL 299 CELLS
2 g/l MICROCARRIERS
PRIOR ART MICROCARRIERS
NEW MICROCARRIERS

FIG. 1.

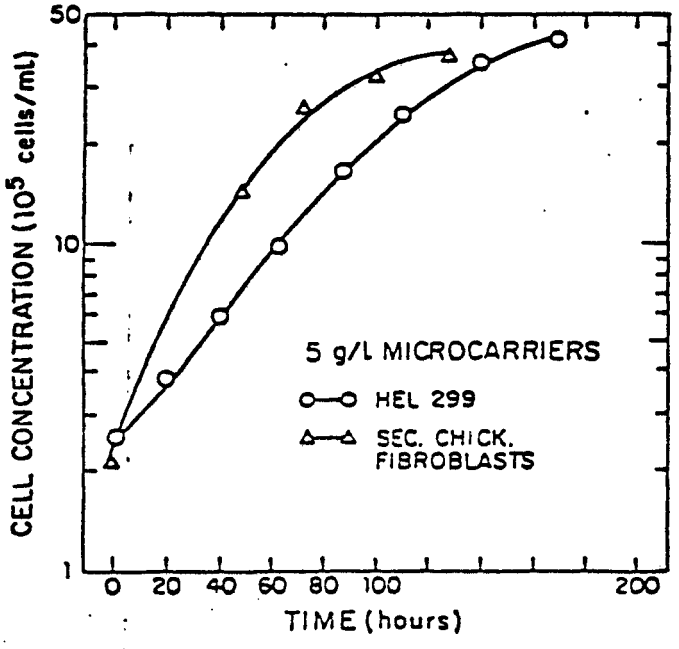

CELL CONCENTRATION (10^5 cells/mL)
50
10
1
0
20
40
60
80
100
200
TIME (hours)
5 g/l MICROCARRIERS
HEL 299
SEC. CHICK. FIBROBLASTS

FIG. 2.

CELL CULTURE MICROCARRIERS

GOVERNMENT SUPPORT

The Government has rights in this invention pursuant to NSF Grant No. BMS 7405676A01 and NIEHS Grant NO. TO1 ES 00063.

RELATED APPLICATION

This is a continuation-in-part of Ser. No. 740,993, filed Nov. 11, 1976 now abandoned.

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention is in the field of biology and more particularly in the field of cell biology.

2. Description of the Prior Art

The ability to grow mammalian cells is important at both the laboratory and industrial levels. At the laboratory level, the limiting factor for cellular or viral research at the sub-cellular level is often the amount of raw material available to be studied. At the industrial level, there is much effort being devoted to the development of pharmaceuticals based on mammalian cell products. These are primarily vaccines for human or animal viruses, but also include human growth hormone and other body hormones and biochemicals for medical applications.

Some mammalian cell types have been adapted for growth in suspension cultures. Examples of such cell types include HeLa (human), BHK (baby hamster kidney) and L cells (mouse). Such cells, in general, have non-normal genetic complements, i.e., too many or too few chromosomes or abnormal chromosomes. Often, these cells will produce a tumor upon injection into an animal of the appropriate species.

Other mammalian cell types have not been adapted for growth in suspension culture to date, and will grow only if they can become attached to an appropriate surface. Such cell types are generally termed "anchorage-dependent" and include 3T3 mouse fibroblasts, mouse bone marrow epithelial cells; Murine leukemia virus-producing strains of mouse fibroblasts, primary and secondary chick fibroblasts; WI-38 human fibroblast cells; and, normal human embryo lung fibroblast cells (HEL299, ATCC #CCL137). Some anchorage-dependent cells have been grown which are tumor causing but others were grown and found to be non-tumor causing. Also, some anchorage-dependent cells, such as WI-38 and HEL299, can be grown which are genetically normal.

Whereas considerable progress has been made in large scale mammalian cell propagation using cell lines capable of growth in suspension culture, progress has been very limited for large scale propagation of anchorage-dependent mammalian cells. Previous operational techniques employed for large scale propagation of anchorage-dependent cells were based on linear expansion of small scale processes. Cell culture plants utilized a large number of low yield batch reactors, in the forms of dishes, prescription bottles, roller tubes and roller bottles. Each of these was a discrete unit or isolated batch reactor requiring individual environmental controls. These controls, however, were of the most primitive type due to economic considerations. Variation in nutrients was corrected by a medium change, an operation requiring two steps, i.e., medium removal and medium addition. Since it was not uncommon for a moderately sized facility to operate hundreds of these batch reactors at a time, even a single change of medium required hundreds of operations, all of which had to be performed accurately, and under exacting sterile conditions. Any multiple step operation, such as cell transfer or harvest, compounded the problem accordingly. Thus, costs of equipment, space and manpower were great for this type of facility.

There are alternative methods to linear scale-up from small batch cultures which have been proposed. Among such alternatives which have been reported in the literature are plastic bags, stacked plates, spiral films, glass bead propagators, artificial capillaries, and microcarriers. Among these, microcarrier systems offer certain outstanding and unique advantages. For example, great increases in the attainable ratio of growth surface to vessel volume (S/V) can be obtained using microcarriers over both traditional and newly developed alternative techniques. The increase in S/V attainable allows the construction of a single-unit homogeneous or quasi-homogeneous batch or semi-batch propagator for high volumetric productivity. Thus, a single stirred tank vessel with simple feedback control for pH and pO_2 presents a homogeneous environment for a large number of cells thereby eliminating the necessity for expensive and space consuming, controlled environment incubators. Also, the total number of operations required per unit of cells produced is drastically reduced. In summary, microcarriers seem to offer economies of capital, space and manpower in the production of anchorage-dependent cells, relative to current production methods.

Microcarriers also offer the advantage of environmental continuity since the cells are grown in one controlled environment. Thus, microcarriers provide the potential for growing anchorage-dependent mammalian cells under one set of environmental conditions which can be regulated to provide constant, optimal cell growth.

One of the more promising microcarrier systems to date has been reported by van Wezel and involves the use of diethylaminoethyl (DEAE)-substituted dextran beads in a stirred tank. A. L. van Wezel, "Growth of Cell Strains and Primary Cells on Microcarriers in Homogeneous Culture", *Nature* 216:64 (1967); D. van Hemert, D. G. Kilburn and A. L. van Wezel, "Homogeneous Cultivation of Animal Cells for the Production of Virus and Virus Products", *Biotechnol. Bioeng.* 11:875 (1969); and A. L. van Wezel, "*Microcarrier Cultures of Animal Cells*", *Tissue Culture, Methods and Applications*, P. F. Kruse and M. K. Patterson, eds., *Academic Press, New York*, p. 372 (1973). These beads are commercially produced by Pharmacia Fine Chemicals, Inc., Piscataway, N.J., under the tradename DEAE-Sephadex A50, an ion exchange system. Chemically, these beads are formed from a crosslinked dextran matrix having diethylaminoethyl groups covalently bound to the dextran chains. As commercially available, DEAE-Sephadex A50 beads are believed to have a particle size of 40–120μm and a positive charge capacity of about 5.4 meq per gram of dry, crosslinked dextran (ignores weight of attached DEAE moieties). Other anion exchange resins, such as DEAE-Sephadex A25, QAE-Sephadex A50 and QAE-Sephadex A25 were also stated by van Wezel to support cell growth.

The system proposed by van Wezel combines multiple surfaces with movable surfaces and has the potential

for innovative cellular manipulations and offers advantages in scale-up and environmental controls. Despite this potential, these suggested techniques have not been significantly exploited because researchers have encountered difficulties in cell production due to certain deleterious effects caused by the beads. Among these are initial cell death among a high percentage of the cell inoculum and inadequate cell growth even for those cells which attach. The reasons for these deleterious effects are not thoroughly understood, although it has been proposed that they may be due to bead toxicity or nutrient adsorption. See van Wezel, A. L. (1967), *Nature* 216: 64–65; van Wezel, A. L. (1973), *Tissue Culture, Methods and Applications.* Kruse, P. R. and Patterson, M. R. (eds.), pp. 372–377, Academic Press, New York; van Hemert, P., Kilburn, D. G., and van Wezel, A. L. (1969), *Biotechnol. Bioeng.* 11: 875–885; Horng, C. and McLimans, W. (1975), *Biotechnol. Bioeng.* 17: 713–732.

It could be that the deleterious effects of these commercially available ion exchange resins are due to their method of manufacture. Certain of these production methods are described for polyhydroxy materials in patents such as: U.S. Pat. Nos. 3,277,025; 3,275,576; 3,042,667 and 3,208,994 all to Flodin et al. Whatever the reason, however, the presently commercially available materials are simply not sufficient for good cell growth of a wide variety of cell types.

One solution to overcoming some of the deleterious effects encountered in attempts to use such commercially available microcarriers for cell growth is described in U.S. Pat. No. 4,036,693, issued on July 19, 1977 to Levine et al. Therein, a method for treating these commercially available ion exchange resins with macromolecular polyanions, such as carboxymethylcellulose, is proposed. While this method has proven successful, it would clearly be more advantageous if the beads could be manufactured initially to have properties designed for outstanding growth of anchorage-dependent cells.

SUMMARY OF THE INVENTION

It has now been discovered that the charge capacity of microcarriers has to be adjusted and/or controlled within a certain range to result in good growth of a wide variety of anchorage-dependent cell types at reasonable microcarrier concentrations. Based upon this discovery, microcarrier beads have been produced with controlled charge capacities and such beads have been used to obtain good growth of a variety of anchorage-dependent cells. Cells grown using such microcarrier systems can be harvested or used in the production of animal or plant viruses, vaccines, hormones, interferon or other cell growth by-products.

One example of the improved microcarriers is those produced using polymers with pendant hydroxy groups, such as crosslinked dextran beads. These beads can be treated with an aqueous solution of a tertiary or quaternary amine, such as diethylaminoethylchloride:-chloride, and an alkaline material, such as sodium hydroxide. The specific charge capacity of the beads is controlled by varying the absolute amounts of the dextran, tertiary amine salt and alkaline material, the ratio of these materials, and/or the time and temperature of treatment.

Microcarriers produced according to this invention can be used in cultures without the high initial cell loss heretofore experienced with commercially available microcarriers. Additionally, attached cells spread and grow to confluence on the beads reaching extremely high cell concentrations in the suspending medium. The concentration of microcarriers in suspension is not limited to very low levels as was customary with the prior art materials, and cell growth appears only to be limited by factors which do not appear to be associated with the microcarriers. Because of this, great increases in the volumetric productivity of cell cultures can be obtained. In short, the potential offered from the use of microcarriers in the growth of cells, and particularly anchorage-dependent cells, can now be realized.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a plot graphically illustrating the growth characteristics of normal diploid human embryo lung fibroblast cells (HEL299) at a microcarrier concentration of 2 grams dry, crosslinked dextran/liter for both commercially available DEAE-treated dextran microcarriers and DEAE-treated microcarriers produced according to this invention;

FIG. 2 graphically illustrates the growth characteristics of both normal diploid human embryo lung fibroblast cells (HEL299) and secondary chicken embryo fibroblasts at a microcarrier concentration of 5 grams dry, crosslinked dextran/liter using improved DEAE-treated microcarriers of this invention.

DESCRIPTION OF PREFERRED EMBODIMENTS

As used herein, the terms "microcarriers", "cell-culture microcarriers" and "cell-growth microcarriers" mean small, discrete particles suitable for cell attachment and growth. Often, although not always, microcarriers are porous beads which are formed from polymers. Usually, cells attach to and grow on the outer surfaces of such beads.

As previously described, it has now been discovered that the amount of charge capacity on cell culture microcarriers must be adjusted and/or controlled to be within a certain range for adequate cell growth at reasonable microcarrier concentrations. Suitable operating and preferred ranges will vary with such factors as the specific cells to be grown, the nature of the microcarriers, the concentration of microcarriers, and other culture parameters including medium composition. In all cases, however, the amount of charge capacity which has been found to be suitable is significantly below the amounts believed to be present on commercially available anion exchange resins previously suggested for microcarrier cell cultures. For example, it is believed that the DEAE-Sephadex A50 beads, suggested by van Wezel, have a charge capacity of about 5.4 meq/gram of dry, untreated (without DEAE), crosslinked dextran. In contradistinction to this relatively high charge capacity, microcarriers have been produced and found suitable for good cell growth according to this invention which have between about 0.1 and about 4.5 meq/-gram of dry, untreated microcarriers. Below about 0.1 meq/gram, it is believed that cells would have difficulty attaching to the microcarriers. Above about 4.5 meq/-gram, losses of initial cell inoculum take place, and even the surviving cells do not grow well, particularly at relatively high microcarrier concentrations.

For the growth of normal diploid human fibroblasts on crosslinked dextran microcarriers, it has been found that a preferred range of charge capacity supplied by DEAE groups is from about 1.0 to about 2.8 meq/gm of dry, untreated crosslinked dextran. While the preferred

range may vary with different cell types or culture conditions, it is believed that the preferred ranges for any given set of conditions will be within the 0.1–4.5 meq/gm range. The preferred and optimum conditions can be determined by a person skilled in the art for any set of conditions by routine experimentation.

It will be recognized, of course, that there are certain deficiencies in attempting to define the charge capacity of microcarriers strictly on a unit weight basis. For example, two beads identical in every way except that they are formed from materials having different densities with the same charge distribution thereon would yield different values for their charge capacity per unit weight. Similarly, two beads having identical charge capacities per unit weight might have quite different charge distributions thereon.

An alternative definition can be made by specifying the range of suitable charges in terms of charge capacity per unit weight of microcarriers in their final functional form. This basis would take into account such factors as the weight of attached DEAE or other positively charged groups, as well as hydration of the beads, etc., whereas the prior definition is based on dry, crosslinked dextran and does not take such factors into account. In an aqueous cell culture medium, the density of microcarriers should be close to 1.0 gram/cc so that the microcarriers can be readily dispersed throughout the culture. Based upon this, it has been determined that the range of suitable charge capacities for microcarriers of this invention defined in this way is from about 0.012 to about 0.25 meq/gram.

The ranges of suitable charge capacities previously specified on a weight basis are valid assuming the microcarriers have a substantially uniform charge distribution throughout their bulk. If the charge distribution is uneven, it might be possible to have suitable microcarriers having charge capacities outside of those ranges. The important criterion is, of course, that the charge capacity be adjusted to and/or controlled at a value sufficient to allow good cell growth on the microcarriers.

Since it may be the charge pattern on the outer surface which is important, it is also desirable to be able to define the suitable charge capacity range in terms of the likely surface pattern. This can be done by assuming that the active portion of the microcarriers represents only the outer surface of the bead to a depth of about 20 angstroms. If it is also assumed that the charged groups in the previously mentioned cases are evenly distributed throughout the beads, the previous ranges can be converted to a charge capacity in this outer shell. Using this approach, the range of charge capacity found suitable is from about 0.012 meq/cm^3 to about 0.25 meq/cm^3. This approach takes changes in microcarrier volume due to different charge densities into account.

Microcarriers having the required charge capacity can be prepared by treating microcarriers formed from polymers containing pendant hydroxyl groups with an aqueous solution of an alkaline material and a tertiary or quaternary amine. The beads can be initially swollen in an aqueous medium without the other ingredients, or can be simply contacted with an aqueous medium containing the required base and amine. This method of using alkaline materials to catalyze the attachment of positively charged amino groups to hydroxyl-containing polymers is described in Hartmann, U.S. Pat. No. 1,777,970.

Examples of suitable hydroxyl-containing polymers include polysaccharides such as dextran, dextrin, starch, cellulose, polyglucose and substituted derivatives of these. Certain synthetic polymers such as polyvinyl alcohol and hydroxy-substituted acrylates or methacrylates, such as hydroxyethyl methacrylate, are also suitable. Dextran, and especially crosslinked dextran in the form of small spheres or beads, is particularly preferred because it is commercially available, relatively inexpensive, and produces microcarriers which support excellent cell growth.

Any material which is alkaline can be used for the reaction. The alkali metal hydroxides, such as sodium or potassium hydroxide, are, however, the preferred alkaline substances.

Either tertiary or quaternary amines are suitable sources of positively charged groups which can be appended onto the hydroxy-containing polymers. Particularly preferred materials are chloro- or bromo-substituted tertiary amines or salts thereof, such as diethylaminoethylchloride, diethylaminoethylbromide, dimethylaminoethylchloride, dimethylaminoethylbromide, diethylaminomethylchloride, diethylaminomethylbromide, di-(hydroxyethyl)-aminoethylchloride, di-(hydroxyethyl)-aminoethylbromide, di-(hydroxyethyl)-aminomethylchloride, di-(hydroxyethyl)-aminomethylbromide, β-morfolinoethylethylchloride, β-morfolinoethylbromide, β-morfolinomethylchloride, β-morfolinomethylbromide and salts thereof, for example, the hydrochlorides.

A wide range of reaction temperatures and times may be used. It is preferred to carry out the reactions at temperatures of about between 18° C. and 65° C. However, other temperatures can be used. The reaction kinetics depend to a large extent, of course, upon the reaction temperature and the concentration of reactants. Both the time and temperature do affect the final exchange capacity achieved.

The reason that the charge capacity of the microcarriers is so critical in cell growth is not thoroughly understood. While not wishing to be bound by this theory, it is possible that the charge capacity at the surface causes certain local discontinuities of medium composition which are the major controlling influence in microcarrier culture cell growth. Nevertheless, this is not meant to rule out other possibilities.

There may be certain beads, of course, that will not be suitable for good cell growth even though they have a charge capacity within one of the ranges specified. This may be due to side chains on the moiety supplying the charge capacity which are toxic or otherwise deleterious for cell growth, the presence of adsorbed or absorbed deleterious compositions or compounds, or it may be due to the porosity of the bead or due to other reasons. If such beads are not suitable for cell growth except for the amount of charge capacity, the beads are not considered to be "cell-growth microcarriers."

The invention is further illustrated by the following examples.

EXAMPLE 1

Preparation of Improved Microcarriers

Improved microcarriers can be produced as follows. Dry, uncharged, crosslinked dextran beads are sieved to obtain those of approximately 75 μm in diameter. One gram of this fraction is added to 10 ml of distilled water and the beads are allowed to swell. An adequate com-

mercial source of dry, crosslinked dextran is Sephadex G-50 from Pharmacia Fine Chemicals, Piscataway, NJ.

An aqueous solution containing 0.01 moles of diethylaminoethylchloride:chloride, twice recrystallized from methylene chloride, and 0.015 moles of sodium hydroxide is formed in a 10 ml volume. This aqueous solution is then added to the swollen dextran bead suspension, which is then agitated vigorously in a shaking water bath for one hour at 60° C. After one hour, the beads are separated from the reaction mixture by filtration on Whatman filter paper No. 595 and washed with 500 ml of distilled water.

Beads made by this procedure contain approximately 2.0 meq of charge capacity per gram of dry, untreated crosslinked dextran. This charge capacity can be characterized by measuring the anion exchange capability of the beads as follows. The bead preparations are washed thoroughly with 0.1 normal HCl to saturate all exchange sites with Cl^- ions. They are then rinsed with 10^{-4} HCl to remove unbound chloride ions. Subsequently, the beads are washed with a 10% (w/w) sodium sulfate solution to countersaturate the exchange sites with $SO_4^=$. The effluent of the sodium sulfate wash is collected and contains liberated chloride ions. This solution is titrated with 1 M silver nitrate using dilute potassium chromate as an indicator.

After titration, the beads are washed thoroughly with distilled water, rinsed with the phosphate-buffered saline solution (PBS), suspended in PBS and autoclaved. This procedure yields hydrated beads of approximately 120–200 μm in diameter, which carry about 2.0 meq of charge capacity per gram of dry, untreated, crosslinked dextran.

EXAMPLE 2

Growth of Anchorage-Dependent Cells With Microcarriers of this Invention Contrasted to Commercially Available Ion Exchange Resin

All cells were grown in Dulbecco's Modified Eagle's Medium. For growth of normal diploid fibroblasts, the medium was supplmented to 10% with fetal calf serum. For growth of primary and secondary chicken fibroblasts, the medium was supplemented with 1% chicken serum, 1% calf serum, and 2% tryptose phosphate broth (Difco Laboratories, Detroit, MI). Stocks were passaged on 100-mm plastic dishes (Falcon Plastics, Inc., Oxnard, CA).

Primary chicken embryo fibroblasts were prepared by mincing and sequentially trypsinizing 10-day embryos. Secondary chicken embryo fibroblasts were prepared on the first day of primary confluence by trypsinization. For cells grown in plastic dishes, doubling time was about 20 hours.

Diploid human fibroblasts derived from embryonic lung (HEL299, ATCC #CCL 137) were obtained from the American Type Culture Collection, Rockville, MD. These cells had a doubling time of 19 hours in plastic dishes.

Microcarrier cultures were initiated simply by combining cells and beads in stirred culture. 100-ml culture volumes in 250-ml glass spinner bottles (6.5 cm in diameter) equipped with a 4.5-cm magnetically driven Teflon ®-coated stir bar (Wilbur Scientific, Inc., Boston, MA) were used. Stirring speed was approximately 90 rpm. Cultures were sampled directly, and samples were examined microscopically and photographed. Cells were enumerated by counting nucleii using the modification of the method of Sanford et al. (Sanford, K. K., Earle, W. R., Evans, V. J., Waltz, H. K., and Shannon, J. E. (1951) *J. Natl Cancer Inst.* 11: 773.) as described by van Wezel (van Wezel, A. L. (1973). *Tissue Culture, Methods and Applications.* Kruse, P. F. and Patterson, M. R. (eds.), pp. 372–377, Academic Press, New York).

Beads with attached cells were separated from the culture medium by permitting the beads to settle at 1 g for a few minutes and then aspirating the supernatant. This procedure greatly facilitated and replacement of medium as well as facilitating the separation of cells from microcarriers after trypsinization.

Commercial DEAE Sephadex A-50 was used as microcarrier for the diploid human fibroblasts and compared with carriers synthesized and titrated as described in Example 1. For both bead types, carrier concentration was 2 grams of dry, untreated, crosslinked dextran per liter. The charge capacity of the DEAE Sephadex A-50 was 5.4 meq/g of dry, crosslinked dextran, while that of the newly synthesized beads was 2.0 meq/g. The results are illustrated in FIG. 1.

For this cell type, loss of original inoculum on A-50 microcarriers was marked, while the fibroblasts attach, proliferate, and reach confluence on the microcarriers of this invention in six days. This behavior agrees well with the reported behavior of this cell type on standard plates. As FIG. 1 shows, the final cell density achieved with the new microcarriers at 2 grams dry, crosslinked dextran/liter was 1.2×10^6 cells/ml.

Cultures containing the new carriers demonstrated neither initial cell loss nor any inhibition in reaching confluence. More importantly, the cultures grew normally at higher microcarrier concentrations. In FIG. 2, for example, human fibroblasts and secondary chicken embryo fibroblasts are shown to reach saturation concentrations near 4×10^6 cells/ml when 5 grams of dry, crosslinked dextran per liter were used with the new carriers having a charge capacity of 2.0 meq/g dextran. As can be seen, even at this relatively high microcarrier concentration, there was no significant loss of inoculum.

Secondary chick embryo fibroblasts were also grown at a microcarrier concentration of 10 grams/liter. With the conditions described above, a saturation concentration of 6×10^6 cells/ml was achieved; with addition to the medium of an additional 1% fetal calf serum, a saturation concentration of 8×10^6 cells/ml was achieved. There was no significant loss of cell inoculum.

Primary chick embryo fibroblasts were grown at a microcarrier concentration of 5 and 10 grams/liter and the growth characteristics were similar to those of the secondary chick fibroblasts, although slight inoculum losses were noted and somewhat longer lag times were encountered.

Attempts were also made to grow secondary chick embryo fibroblasts under conditions similar to those used above except that DEAE-Sephadex A-50 microcarriers at concentrations of 1 and 5 grams/liter were used. No cell growth was detected and significant inoculum loss occurred.

EXAMPLE 3

Preparation of Microcarriers with varying amounts of reactants

Batches of microcarriers were prepared by dissolving diethylaminoethylchloride:chloride and sodium hydroxide in 20 ml of distilled water. The solution was then poured over dry Sephadex G-50 beads after which

the beads were placed on a reciprocating shaker-water table maintained at 60° C. One set of bead batches were treated with a solution containing 0.01 moles of the amine and 0.015 moles of sodium hydroxide, whereas another set of batches was treated with a solution containing 0.03 moles of the amine and 0.045 moles of sodium hydroxide. The reaction time was varied to produce different meq/g within each batch.

Diploid humam fibroblasts (HEL299) were grown in suspension cultures at a microcarrier concentration of 5.0 grams dry, untreated crosslinked dextran per liter following the procedures of Example 2 using microcarriers having varying meq/gram selected from each batch. Subsequently, productivity (10^6 cells grown/liter hour) was calculated and plotted versus meq/gram for each batch of beads produced as above. Curves plotted using data obtained for both sets were similar in shape, having a general bell shape, but the curve from the batches treated with the higher concentration of reactants had a somewhat sharper rise and fall. Carriers yielding excellent cell growth were produced from each batch.

EXAMPLE 4

Preparation of Microcarriers at varying Amine/Alkali Ratios

This example illustrates further changes in the charge capacity which can be obtained by varying DEAE chloride: chloride/NaOH ratios. In this example, the procedures of Example 3 were followed except that a wide range of concentrations of sodium hydroxide was used while maintaining the concentration of the diethylaminoethylchloride:chloride at 0.01 moles per 20 ml. The concentrations used for the sodium hydroxide were 0.01, 0.011, 0.012, 0.013, 0.014, 0.015, 0.02, 0.03, 0.05, 0.75, 0.10 moles per 20 ml.

A plot was made of meq/gram after 1.25 hours at 60° C. versus concentration of sodium hydroxide. It was observed from the plot that concentrations of sodium hydroxide below about 0.01 produced no detectable charge capacity. Charge capacity rose quickly, however, with increases in concentration and reached a maximum of around 2.3 meq/gram dry, crosslinked dextran at a concentration of about 0.014 moles sodium hydroxide. Charge capacity then declined in an almost linear relationship to a value of about 1.1 meq/gram at a sodium hydroxide concentration of about 0.10 moles. Thus, a change in reaction kinetics takes place when the ratio of DEAE Chloride:chloride to sodium hydroxide is varied at a constant concentration of DEAE chloride:chloride and crosslinked dextran.

EXAMPLE 5

Human Interferon Production in Cells grown on improved Microcarriers

The ability of microcarrier grown cells to produce human interferon is described herein. Cells used for the production of human interferon were normal diploid human foreskin fibroblasts, FS-4. These fibroblasts were grown in microcarrier cultures using procedures as in Example 2. Microcarriers prepared and titrated according to Example 1 were used at a concentration of 5 grams of dry, crosslinked dextran/liter. The medium used for culture growth was DMEM supplemented with 10% fetal calf serum.

In 8 to 10 days, cultures ceased growing. At this point, growth medium was removed. Cultures were washed 1–4 times with 100 ml of serum-free DMEM. The cells were then ready for interferon induction. This was accomplished by adding to the cultures 50 ml of serum-free DMEM medium containing 50 μg/ml cyclohexamide, and varying amounts of poly I. poly C inducer. After 4 hours, Actinomycin D was added to the cultures to a final concentration of 1 μg/ml.

Five hours after the onset of induction, inducing medium was decanted and cultures were washed 3–4 times with 100 ml of warm serum-free DMEM. Cultures were replenished with 50 ml of DMEM containing 0.5% human plasma protein. Cultures were incubated under standard conditions for an additional 18 hours. At this time, cultures were decanted, and the decanted medium was assayed for interferon activity. Interferon activity was assayed by determining the 50% level of cell protection for samples and standard solutions, for FS-4 fibroblasts challenged with Vesicular Stomatitis Virus (VSV), Indiana strain. The results of interferon production runs are presented in tabular form below.

Inducer Concentration (μg/ml)	Cell Concentration During Production (cells/ml)	Interferon (U/10^6 cells)
4	2.0×10^6	39
5	2.6×10^6	378
25	2.6×10^6	886
50	2.0×10^6	~5000

these data are each from a separate run and are not intended to demonstrate any correlation to inducer concentration.

EXAMPLE 6

Growth of Cells on Improved Microcarriers for The Purpose of Producing Viruses

The ability of microcarrier grown cells to produce a virus is described here. Primary and secondary chicken embryo fibroblasts were grown in microcarrier culture according to the procedure described in Example 2 with the primary cells grown at 10 grams/liter and the secondary at 5 grams/liter microcarrier concentration. To initiate virus production, growth medium was removed, and the cultures were washed twice with 100 ml of serum free DMEM. Infection of cells with Sindbis virus took place in 50 ml of DMEM supplemented with 1% calf serum, 2% tryptose phosphate broth, and enough Sindbis virus to equal an MOI (multiplicity of infection) of 0.05.

The virus was harvested 24 hours after infection, by collecting culture broth, clarifying at low centrifugation, and freezing the supernatant. Virus production was assayed by plaque formation in a field of secondary chicken fibroblasts. The results of infecting these microcarrier cultures were:

Cell Type	All Concentration For Production (cells/ml)	(PFU/ml)	PFU/cell
Secondary	4.0×10^6	5.4×10^9	2,100
Primary	1.4×10^6	2.3×10^{10}	16,000
Primary	6.0×10^6	2.6×10^{10}	5,000

Virus production was also established for the following virus/cell on microcarrier combinations: Polio/WI-

38; Moloney MuLV/Cl-1 mouse and VSV/chick embryo fibroblasts.

EXAMPLE 7

Comparative Growth of Cells in Roller Bottles and with Improved Microcarriers for the Purpose of Producing Murine Leukemia Virus Proviral DNA

The reverse-transcribed DNA of Moloney leukemia virus (M-MuLV) after infection of JLS-V9 cells, a mouse bone marrow line, was studied.

One technique involved growing cells in roller bottles. Cells were grown in roller bottle culture, the medium removed, and virus inoculum introduced into the bottles. Shortly thereafter, the cultures were fed with fresh medium, and 8-16 hours later extracted for eventual purification of viral DNA. The cultures were washed with fresh buffer and the cell lysed with a solution containing the detergent sodium dodecylsulfate. Subsequent cooling of the lysate and addition of salt to one molar caused coprecipitation of the detergent with high molecular weight DNA. The low molecular weight DNA remaining in the supernatant could then be deproteinized and concentrated for further analysis.

A 50-roller bottle culture contained about 10^9 cells. These were infected with about one-liter of viral inoculum titering at 3×10^6 plaque-forming units per ml. This resulted in a nominal multiplicity of infection of 1-3 and the infected cells yielded 5-20 nanograms of virus-specific DNA.

A simpler procedure was developed employing improved microcarriers according to this invention. A culture containing 10 grams of beads in one liter of growth medium was used. Upon reaching confluence, the 10^9 cells on the beads were infected by allowing the beads to settle out and replacing the medium with 1 liter of virus inoculum. For extraction, the cells on the beads were washed with buffer and then placed in the SDS containing buffer. After co-precipitation of the high molecular weight DNA with the detergent, the precipitate together with the beads were centrifuged out and a supernatant extracted for further analysis. The yield of viral DNA was comparable to that obtained in roller bottle culture and the labor involved was 5-10% of that required by roller bottle culture.

EXAMPLE 8

Improved Microcarrier Production with Dimethylaminoethyl Charge Groups

A suitable microcarrier was produced by binding an alternate exchange moiety to the dextran matrix utilized in Example 1. Dimethylaminoethyl groups (DMAE) were bound to a dextran matrix by the following procedure: 1 gm of dextran beads (Pharmacia G-50), 50-75 μm in diameter, dry, was added to 10 ml of distilled water and the beads were allowed to swell. An aqueous solution containing 0.01 moles of dimethylaminoethyl-chloride:chloride (Sigma Chemical Co.) and 0.015 moles of sodium hydroxide was formed in a 10 ml volume. This aqueous solution was added to the swollen dextran beads and this suspension was then agitated vigorously for one hour at 60° C. After reaction, the bead mass was titrated as in Example 1. This reaction binds 1.0 meq of dimethylaminoethyl to the dextran mass. To produce microcarriers of greater degrees of substitution, the above reaction was carried out, and the bead mass washed thoroughly with water. With excess water filtered off, the bead mass was weighed so as to determine the amount of water being retained by the bead mass. To this bead mass was added the appropriate amount of fresh reagents (i.e., DMAE-CL:CL, and NaOH) so that the final concentration of DMAE, and NaOH in these succeeding reaction mixtures were identical to those initially used.

In this manner, a series of microcarriers were prepared at 1.0, 2.0, 2.5 and 3.5 meq DMAE/gm unreacted dextran. Cells (HEL 299) were grown in microcarrier culture (5 gm/l) with these microcarriers according to the procedures in Example 2. The results are tabulated in the following table:

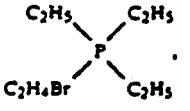
Degree of Substitution (meq/gm)	Cell Spreading	Net Growth
1.0	−	−
2.0	−	−
2.5	+	+
3.2	+	−

As expected, cell growth is related to the degree of substitution with charge carrying groups. At too high a degree of substitution, no cell growth occurs, although attachment and spreading takes place. At too low a degree of substitution, cell adhesion to the surface is not sufficient to allow proper spreading and growth.

EXAMPLE 9

Improved Microcarriers Having Positively Charged Phosphonium Groups

Improved microcarriers were also prepared using non-amine exchange groups as follows. One gram of dry dextran beads were prepared and swollen with water as in Example 1. To the swollen beads were added 5 ml of a saturated aqueous solution of triethyl-(ethyl-bromide)-phosphonium (TEP),



and 5 ml of a 3 molar solution of sodium hydroxide. This slurry was reacted at 65° C. A series of microcarriers were prepared at 1.1, 1.7 and 2.9 meq/gm. Th microcarriers at 1.1 meq/gm were prepared by reaction at the above conditions for 4 minutes. The 1.7 meq/gm microcarriers was reacted for 1 hour, and the 2.9 meq/gm microcarriers were reacted successively 3 times as described in Example 7. A microcarrier cell culture at 5 gm/liter was established for each of these carriers with a continuous cell type, JLS-V9 and compared to this cell's growth on improved DEAE-microcarriers prepared as in Example 3. The results are tabulated in the following table.

meq/gram	Cell Attachment and Spreading	Net Growth
	DEAE	
0.9	+	+
1.7	+	+
3.8	+	−
	TEP	
1.1	+	+
1.7	+	+

-continued

2.9	+	−

It will be recognized by those skilled in the art that there are certain equivalents to the specific techniques, materials, etc., described herein, and these are considered to be part of this invention and are intended to be covered by the following claims. Additionally, while most of the description herein has been limited to the use of the improved microcarriers for growth of anchorage-dependent cells, they can also be used, of course, for the growth of other cell types.

What is claimed is:

1. In a method of growing anchorage-dependent cells in microcarrier culture, the improvement of employing microcarriers having an amount of positively charged chemical moieties thereon adjusted to provide an exchange capacity which allows good growth of said cells, said exchange capacity being within the range of between about 0.1 and about 4.5 meq/gram of dry, untreated microcarriers.

2. In the method of claim 1, the improvement wherein said microcarriers comprise crosslinked dextran beads.

3. In the method of claim 2, the improvement wherein the positively charged chemical moieties on said crosslinked dextran beads comprise tertiary or quaternary amine groups.

4. In the method of claim 3, the improvement wherein said exchange capacity is within the range of from about 1 to about 2.8 meq/gram of dry, untreated crosslinked dextran.

5. In the method of claim 4, the improvement wherein the positively charged chemical moieties on said crosslinked dextran beads comprise diethylaminoethyl groups.

6. In the method of claim 5, the improvement wherein said dry, crosslinked beads have a diameter of approximately 75 μm in their dry state.

7. A method of growing anchorage-dependent cells, comprising:

a. forming a suspension in cell culture medium of positively charged microcarriers having their charge capacity adjusted to a value which supports good growth of cells, said charge capacity being within the range of from about 0.1 to about 4.5 meq/gram of dry, untreated microcarriers;

b. inoculating cells into said suspension of microcarriers to form a cell culture; and,

c. maintaining said cell culture under cell growth conditions.

8. A method of claim 7 wherein said microcarriers comprise crosslinked dextran beads having tertiary or quaternary amine groups thereon.

9. A method of claim 7 wherein said microcarriers comprise crosslinked dextran beads having diethylaminoethyl groups thereon.

10. A method of claim 9 wherein said microcarriers have a charge capacity within the range of between about 1 and about 2.8 meq/gram of dry, untreated crosslinked dextran.

11. A method of claim 10 wherein said microcarriers have an average diameter of about 75 μm.

12. In the method of growing anchorage-dependent cells including forming a suspension in cell culture medium of microcarriers comprising crosslinked dextran beads, inoculating cells into said suspension to form a cell culture and maintaining said cell culture under cell growth conditions:

The improvement of pre-treating said crosslinked dextran beads by reacting them in an aqueous solution of a tertiary or quaternary amine and a base under conditions sufficient to provide a positive charge capacity on said beads which is suitable for exponential growth of said anchorage-dependent cells, said charge capacity being within the range between about 0.1 and about 4.5 meq/gram of dry, untreated microcarriers.

13. A method for producing anchorage-dependent cell growth by-products comprising:

a. forming a suspension of positively-charged microcarriers having a charge capacity sufficient for good growth of anchorage dependent cells in a suitable cell culture medium, said charge capacity being between about 0.1 and about 4.5 meq/gram of dry, untreated microcarriers;

b. inoculating said culture with anchorage-dependent cells to form a cell culture;

c. maintaining said cell culture under conditions conducive to the production of cell growth by-products; and,

d. harvesting said cell growth by-products.

14. A method of claim 13 wherein said cell growth by-product is a virus.

15. A method of claim 13 wherein said cell growth by-product is a hormone.

16. A method of claim 13 wherein said cell growth by-product is interferon.

17. A method of claim 13 wherein said microcarriers comprise a reaction product of crosslinked dextran beads and an aqueous solution of a tertiary or quaternary amine and a base.

18. A method of claim 13 wherein said microcarriers comprise crosslinked dextran beads having diethylaminoethyl groups thereon.

19. A method of claim 18 wherein said charge capacity is in the range of from about 1 to about 2.8 meq/grams of dry, untreated microcarriers.

20. A method of claim 19 wherein said reaction product comprises hydrated beads having an average diameter of about 120–200 μm.

* * * * *

United States Patent [19]

Levine et al.

[11] 4,293,654

[45] Oct. 6, 1981

[54] CELL CULTURE MICROCARRIERS

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[73] Assignee: Massachusetts Institute of Technology, Cambridge, Mass.

[21] Appl. No.: 54,319

[22] Filed: Jul. 2, 1979

Related U.S. Application Data

[60] Division of Ser. No. 842,696, Oct. 17, 1977, Pat. No. 4,189,534, which is a continuation-in-part of Ser. No. 740,993, Nov. 11, 1976, abandoned.

[51] Int. Cl.³ C12N 5/02; C08B 37/00

[52] U.S. Cl. 435/241; 525/61; 525/379; 536/18; 536/30; 536/45; 536/51; 536/112

[58] Field of Search 536/51, 112, 45, 30, 536/18; 525/61, 379; 435/241, 240

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Primary Examiner—Sam Rosen

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[57] ABSTRACT

Improved cell culture microcarriers, and methods for their production and use, are disclosed herein. These improved microcarriers have positive charge capacities adjusted and/or controlled within a range suitable for good cell growth. One method for producing such improved microcarriers is by treating beads formed from polymers containing pendant hydroxy groups, such as dextran beads, with an aqueous solution of an alkaline material and a chloro- or bromo-substituted tertiary amine under precisely controlled conditions to produce the desired exchange capacity. The resultant positively charged microcarriers have been used in microcarrier cultures to produce outstanding growth of anchorage-dependent cells. Such cells can be harvested, or used for the production of viruses, vaccines, hormones, interferon or other cellular growth by-products.

9 Claims, 2 Drawing Figures

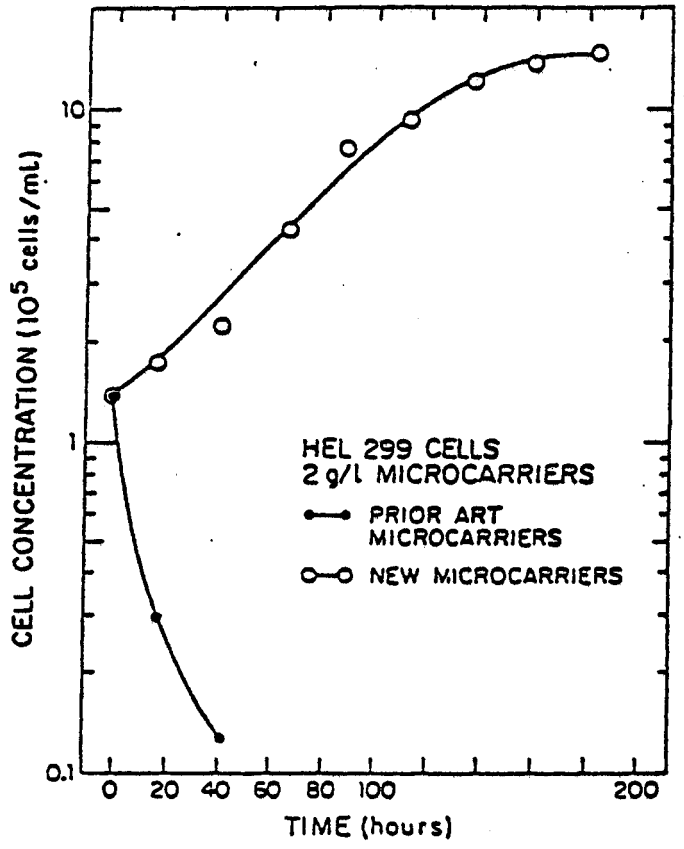

CELL CONCENTRATION (10^5 cells/mL)
10
1
0.1
0 20 40 60 80 100 200
TIME (hours)
HEL 299 CELLS
2 g/l MICROCARRIERS
PRIOR ART MICROCARRIERS
NEW MICROCARRIERS

FIG. 1.

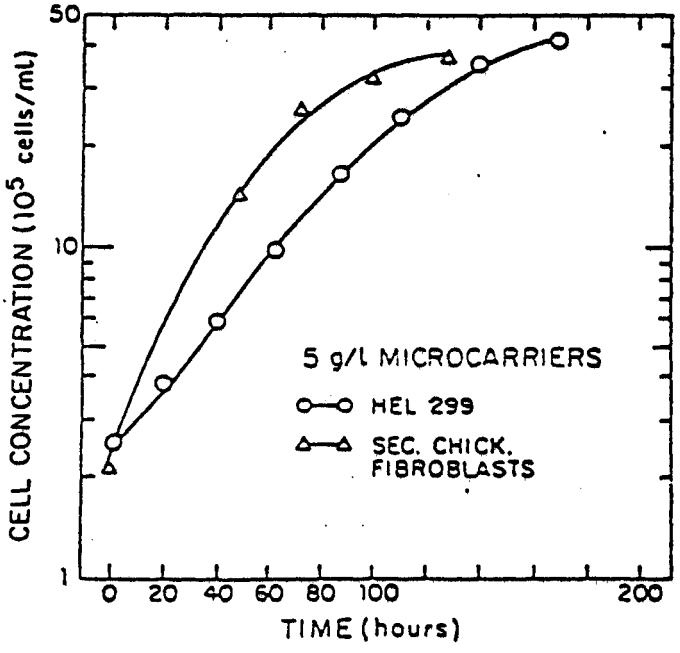

CELL CONCENTRATION (10^5 cells/mL)
50
10
1
0 20 40 60 80 100 200
TIME (hours)
5 g/l MICROCARRIERS
HEL 299
SEC. CHICK. FIBROBLASTS

FIG. 2.

CELL CULTURE MICROCARRIERS

GOVERNMENT SUPPORT

The Government has rights in this invention pursuant to NSF Grant No. BMS 7405676A01 and NIEHS Grant No. TO1ES 00063.

RELATED APPLICATION

This is a division, of application Ser. No. 842,696, filed Oct. 17, 1977, now U.S. Pat. No. 4,189,534 which in turn is a continuation-in-part of Ser. No. 740,993, filed Nov. 11, 1976, now abandoned.

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention is in the field of biology and more particularly in the field of cell biology.

2. Description of the Prior Art

The ability to grow mammalian cells is important at both the laboratory and industrial levels. At the laboratory level, the limiting factor for cellular or viral research at the sub-cellular level is often the amount of raw material available to be studied. At the industrial level, there is much effort being devoted to the development of pharmaceuticals based on mammalian cell products. These are primarily vaccines for human or animal viruses, but also include human growth hormone and other body hormones and biochemicals for medical applications.

Some mammalian cell types have been adapted for growth in suspension cultures. Examples of such cell types include HeLa (human), BHK (baby hamster kidney) and L cells (mouse). Such cells, in general, have non-normal genetic complements, i.e., too many or too few chromosomes or abnormal chromosomes. Often, these cells will produce a tumor upon injection into an animal of the appropriate species.

Other mammalian cell types have not been adapted for growth in suspension culture to date, and will grow only if they can become attached to an appropriate surface. Such cell types are generally termed "anchorage-dependent" and include 3T3 mouse fibroblasts, mouse bone marrow epithelial cells; Murine leukemia virus-producing strains of mouse fibroblasts, primary and secondary chick fibroblasts; WI-38 human fibroblast cells; and, normal human embryo lung fibroblast cells (HEL299, ATCC #CCL137). Some anchorage-dependent cells have been grown which are tumor causing but others were grown and found to be non-tumor causing. Also, some anchorage-dependent cells, such as WI-38 and HEL299, can be grown which are genetically normal.

Whereas considerable progress has been made in large scale mammalian cell propagation using cell lines capable of growth in suspension culture, progress has been very limited for large scale propagation of anchorage-dependent mammalian cells. Previous operational techniques employed for large scale propagation of anchorage-dependent cells were based on linear expansion of small scale processes. Cell culture plants utilized a large number of low yield batch reactors, in the forms of dishes, prescription bottles, roller tubes and roller bottles. Each of these was a discrete unit or isolated batch reactor requiring individual environmental controls. These controls, however, were of the most primitive type due to economic considerations. Variation in nutrients was corrected by a medium change, an operation requiring two steps, i.e., medium removal and medium addition. Since it was not uncommon for a moderately sized facility to operate hundreds of these batch reactors at a time, even a single change of medium required hundreds of operations, all of which had to be performed accurately, and under exacting sterile conditions. Any multiple step operation, such as cell transfer or harvest, compounded the problem accordingly. Thus, costs of equipment, space and manpower were great for this type of facility.

There are alternative methods to linear scale-up from small batch cultures which have been proposed. Among such alternatives which have been reported in the literature are plastic bags, stacked plates, spiral films, glass bead propagators, artificial capillaries, and microcarriers. Among these, microcarrier systems offer certain outstanding and unique advantages. For example, great increases in the attainable ratio of growth surface to vessel volume (S/V) can be obtained using microcarriers over both traditional and newly developed alternative techniques. The increase in S/V attainable allows the construction of a single-unit homogeneous or quasi-homogeneous batch or semi-batch propagator for high volumetric productivity. Thus, a single stirred tank vessel with simple feedback control for pH and pO_2 presents a homogeneous environment for a large number of cells thereby eliminating the necessity for expensive and space consuming, controlled environment incubators. Also, the total number of operations required per unit of cells produced is drastically reduced. In summary, microcarriers seem to offer economies of capital, space and manpower in the production of anchorage-dependent cells, relative to current production methods.

Microcarriers also offer the advantage of environmental continuity since the cells are grown in one controlled environment. Thus, microcarriers provide the potential for growing anchorage-dependent mammalian cells under one set of environmental conditions which can be regulated to provide constant, optimal cell growth.

One of the more promising microcarrier systems to date has been reported by van Wezel and involves the use of diethylaminoethyl (DEAE)-substituted dextran beads in a stirred tank. A. L. van Wezel, "Growth of Cell Strains and Primary Cells on Microcarriers in Homogeneous Culture," *Nature* 216:64 (1967); D. van Hemert, D. G. Kilburn and A. L. van Wezel, "Homogeneous Cultivation of Animal Cells for the Production of Virus and Virus Products," *Biotechnol. Bioeng.* 11:875 (1969); and A. L. van Wezel, "Microcarrier Cultures of Animal Cells," *Tissue Culture, Methods and Applications*, P. F. Kruse and M. K. Patterson, eds., Academic Press, New York, p. 372 (1973). These beads are commercially produced by Pharmacia Fine Chemicals, Inc., Piscataway, N.J., under the tradename DEAE-Sephadex A50, an ion exchange system. Chemically, these beads are formed from a crosslinked dextran matrix having diethylaminoethyl groups covalently bound to the dextran chains. As commercially available, DEAE-Sephadex A50 beads are believed to have a particle size of 40–120 μm and a positive charge capacity of about 5.4 meq per gram of dry, crosslinked dextran (ignores weight of attached DEAE moieties). Other anion exchange resins, such as DEAE-Sephadex A25, QAE-Sephadex A50 and QAE-Sephadex A25 were also stated by van Wezel to support cell growth.

The system proposed by van Wezel combines multiple surfaces with movable surfaces and has the potential for innovative cellular manipulations and offers advantages in scale-up and environmental controls. Despite this potential, these suggested techniques have not been significantly exploited because researchers have encountered difficulties in cell production due to certain deleterious effects caused by the beads. Among these are initial cell death among a high percentage of the cell inoculum and inadequate cell growth even for those cells which attach. The reasons for these deleterious effects are not thoroughly understood, although it has been proposed that they may be due to bead toxicity or nutrient adsorption. See van Wezel, A. L. (1967), *Nature* 216: 64–65; van Wezel, A. L. (1973), *Tissue Culture, Methods and Applications.* Kruse, P. R. and Patterson, M. R. (eds.), pp. 372–377, Academic Press, New York; van Hemert, P., Kilburn, D. G., and van Wezel, A. L. (1969), *Biotechnol. Bioeng.* 11: 875–885; Horng, C. and McLimans, W. (1975), *Biotechnol. Bioeng.* 17: 713–732.

It could be that the deleterious effects of these commercially available ion exchange resins are due to their method of manufacture. Certain of these production methods are described for polyhydroxy materials in patents such as: U.S. Pat. Nos. 3,277,025; 3,275,576; 3,042,667 and 3,208,994 all to Flodin et al. Whatever the reason, however, the presently commercially available materials are simply not sufficient for good cell growth of a wide variety of cell types.

One solution to overcoming some of the deleterious effects encountered in attempts to use such commercially available microcarriers for cell growth is described in U.S. Pat. No. 4,036,693, issued on July 19, 1977 to Levine et al. Therein, a method for treating these commercially available ion exchange resins with macromolecular polyanions, such as carboxymethylcellulose, is proposed. While this method has proven successful, it would clearly be more advantageous if the beads could be manufactured initially to have properties designed for outstanding growth of anchorage-dependent cells.

SUMMARY OF THE INVENTION

It has now been discovered that the charge capacity of microcarriers has to be adjusted and/or controlled within a certain range to result in good growth of a wide variety of anchorage-dependent cell types at reasonable microcarrier concentrations. Based upon this discovery, microcarrier beads have been produced with controlled charge capacities and such beads have been used to obtain good growth of a variety of anchorage-dependent cells. Cells grown using such microcarrier systems can be harvested or used in the production of animal or plant viruses, vaccines, hormones, interferon or other cell growth by-products.

One example of the improved microcarriers is those produced using polymers with pendant hydroxy groups, such as crosslinked dextran beads. These beads can be treated with an aqueous solution of a tertiary or quaternary amine, such as diethylaminoethylchloride:-chloride, and an alkaline material, such as sodium hydroxide. The specific charge capacity of the beads is controlled by varying the absolute amounts of the dextran, tertiary amine salt and alkalne material, the ratio of these materials, and/or the time and temperature of treatment.

Microcarriers produced according to this invention can be used in cultures without the high initial cell loss heretofore experienced with commercially available microcarriers. Additionally, attached cells spread and grow to confluence on the beads reaching extremely high cell concentrations in the suspending medium. The concentration of microcarriers in suspension is not limited to very low levels as was customary with the prior art materials, and cell growth appears only to be limited by factors which do not appear to be associated with the microcarriers. Because of this, great increases in the volumetric productivity of cell cultures can be obtained. In short, the potential offered from the use of microcarriers in the growth of cells, and particularly anchorage-dependent cells, can now be realized.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a plot graphically illustrating the growth characteristics of normal diploid human embryo lung fibroblast cells (HEL299) at a microcarrier concentration of 2 grams dry, crosslinked dextran/liter for both commercially available DEAE-treated dextran microcarriers and DEAE-treated microcarriers produced according to this invention;

FIG. 2 graphically illustrates the growth characteristics of both normal diploid human embryo lung fibroblast cells (HEL299) and secondary chicken embryo fibroblasts at a microcarrier concentration of 5 grams dry, crosslinked dextran/liter using improved DEAE-treated microcarriers of this invention.

DESCRIPTION OF PREFERRED EMBODIMENTS

As used herein, the terms "microcarriers," "cell-culture microcarriers" and "cell-growth microcarriers" mean small, discrete particles suitable for cell attachment and growth. Often, although not always, microcarriers are porous beads which are formed from polymers. Usually, cells attach to and grow on the outer surfaces of such beads.

As previously described, it has now been discovered that the amount of charge capacity on cell culture microcarriers must be adjusted and/or controlled to be within a certain range for adequate cell growth at reasonable microcarrier concentrations. Suitable operating and preferred ranges will vary with such factors as the specific cells to be grown, the nature of the microcarriers, the concentration of microcarriers, and other culture parameters including medium composition. In all cases, however, the amount of charge capacity which has been found to be suitable is significantly below the amounts believed to be present on commercially available anion exchange resins previously suggested for microcarrier cell cultures. For example, it is believed that the DEAE-Sephadex A50 beads, suggested by van Wezel, have a charge capacity of about 5.4 meq/gram of dry, untreated (without DEAE), crosslinked dextran. In contradistinction to this relatively high charge capacity, microcarriers have been produced and found suitable for good cell growth according to this invention which have between about 0.1 and about 4.5 meq/-gram of dry, untreated microcarriers. Below about 0.1 meq/gram, it is believed that cells would have difficulty attaching to the microcarriers. Above about 4.5 meq/-gram, losses of initial cell inoculum take place, and even the surviving cells do not grow well, particularly at relatively high microcarrier concentrations.

For the growth of normal diploid human fibroblasts on crosslinked dextran microcarriers, it has been found that a preferred range of charge capacity supplied by

gram of this fraction is added to 10 ml of distilled water and the beads are allowed to swell. An adequate commercial source of dry, crosslinked dextran is Sephadex G-50 from Pharmacia Fine Chemicals, Piscataway, N.J.

An aqueous solution containing 0.01 moles of diethylaminoethylchloride:chloride, twice recrystallized from methylene chloride, and 0.015 moles of sodium hydroxide is formed in a 10 ml volume. This aqueous solution is then added to the swollen dextran bead suspension, which is then agitated vigorously in a shaking water bath for one hour at 60° C. After one hour, the beads are separated from the reaction mixture by filtration on Whatman filter paper No. 595 and washed with 500 ml of distilled water.

Beads made by this procedure contain approximately 2.0 meq of charge capacity per gram of dry, untreated cross-linked dextran. This charge capacity can be characterized by measuring the anion exchange capability of the beads as follows. The bead preparations are washed thoroughly with 0.1 normal HCl to saturate all exchange sites with Cl^- ions. They are then rinsed with 10^{-4} normal HCl to remove unbound chloride ions. Subsequently, the beads are washed with a 10% (w/w) sodium sulfate solution to countersaturate the exchange sites with $SO_4=$. The effluent of the sodium sulfate wash is collected and contains liberated chloride ions. This solution is titrated with 1 M silver nitrate using dilute potassium chromate as an indicator.

After titration, the beads are washed thoroughly with distilled water, rinsed with the phosphate-buffered saline solution (PBS), suspended in PBS and autoclaved. This procedure yields hydrated beads of approximately 120–200 μm in diameter, which carry about 2.0 meq of charge capacity per gram of dry, untreated, crosslinked dextran.

EXAMPLE 2

Growth of Anchorage-Dependent Cells With Microcarriers of this Invention Contrasted to Commercially Available Ion Exchange Resin

All cells were grown in Dulbecco's Modified Eagle's Medium. For growth of normal diploid fibroblasts, the medium was supplemented to 10% with fetal calf serum. For growth of primary and secondary chicken fibroblasts, the medium was supplemented with 1% chicken serum, 1% calf serum, and 2% tryptose phosphate broth (Difco Laboratories, Detroit, MI). Stocks were passaged on 100-mm plastic dishes (Falcon Plastics, Inc., Oxnard, CA).

Primary chicken embryo fibroblasts were prepared by mincing and sequentially trypsinizing 10-day embryos. Secondary chicken embryo fibroblasts were prepared on the first day of primary confluence by trypsinization. For cells grown in plastic dishes, doubling time was about 20 hours.

Diploid human fibroblasts derived from embryonic lung (HEL299, ATCC #CCL 137) were obtained from the American Type Culture Collection, Rockville, MD. These cells had a doubling time of 19 hours in plastic dishes.

Microcarrier cultures were initiated simply by combining cells and beads in stirred culture. 100-ml culture volumes in 250-ml glass spinner bottles (6.5 cm in diameter) equipped with a 4.5-cm magnetically driven Teflon® coated air bar (Wilbur Scientific, Inc., Boston, MA) were used. Stirring speed was approximately 90 rpm. Cultures were sampled directly, and samples were examined microscopically and photographed. Cells were enumerated by counting nuclei using the modification of the method of Sanford et al. (Sanford, K. K., Earle, W. R., Evans, V. J., Waltz, H. K., and Shannon, J. E. (1951) *J. Natl Cancer Inst.* 11: 773.) as described by van Wezel (van Wezel, A. L. (1973). *Tissue Culture, Methods and Applications.* Kruse, P. F. and Patterson, M. R. (eds), pp. 372—377, Academic Press. New York).

Beads with attached cells were separated from the culture medium by permitting the beads to settle at 1 g for a few minutes and then aspirating the supernatant. This procedure greatly facilitated the replacement of medium as well as facilitating the separation of cells from microcarriers after trypsinization.

Commercial DEAE Sephadex A-50 was used as microcarrier for the diploid human fibroblasts and compared with carriers synthesized and titrated as described in Example 1. For both bead types, carrier concentration was 2 grams of dry, untreated, crosslinked dextran per liter. The charge capacity of the DEAE Sephadex A-50 was 5.4 meq/g of dry, crosslinked dextran, while that of the newly synthesized beads was 2.0 meq/g. The results are illustrated in FIG. 1.

For this cell type, loss of original inoculum on A-50 microcarriers was marked, while the fibroblasts attach, proliferate, and reach confluence on the microcarriers of this invention in six days. This behavior agrees well with the reported behavior of this cell type on standard plates. As FIG. 1 shows, the final cell density achieved with the new microcarriers at 2 grams dry, crosslinked dextran/liter was 1.2×10^6 cells/ml.

Cultures containing the new carriers demonstrated neither initial cell loss nor any inhibition in reaching confluence. More importantly, the cultures grew normally at higher microcarrier concentrations. In FIG. 2, for example, human fibroblasts and secondary chicken embryo fibroblasts are shown to reach saturation concentrations near 4×10^6 cells/ml when 5 grams of dry, crosslinked dextran per liter were used with the new carriers having a charge capacity of 2.0 meq/g dextran. As can be seen, even at this relatively high microcarrier concentration, there was no significant loss of inoculum.

Secondary chick embryo fibroblasts were also grown at a microcarrier concentration of 10 grams/liter. With the conditions described above, a saturation concentration of 6×10^6 cells/ml was achieved; with addition to the medium of an additional 1% fetal calf serum, a saturation concentration of 8×10^6 cells/ml was achieved. There was no significant loss of cell inoculum.

Primary chick embryo fibroblasts were grown at a microcarrier concentration of 5 and 10 grams/liter and the growth characteristics were similar to those of the secondary chick fibroblasts, although slight inoculum losses were noted and somewhat longer lag times were encountered.

Attempts were also made to grow secondary chick embryo fibroblasts under conditions similar to those used above except that DEAE-Sephadex A-50 microcarriers at concentrations of 1 and 5 grams/liter were used. No cell growth was detected and significant inoculum loss occurred.

EXAMPLE 3

Preparation of Microcarriers With Varying Amounts of Reactants

Batches of microcarriers were prepared by dissolving diethylaminoethylchloride:chloride and sodium hy-

DEAE groups is from about 1.0 to about 2.8 meq/gm of dry, untreated crosslinked dextran. While the preferred range may vary with different cell types or culture conditions, it is believed that the preferred ranges for any given set of conditions will be within the 0.1–4.5 meq/gm range. The preferred and optimum conditions can be determined by a person skilled in the art for any set of conditions by routine experimentation.

It will be recognized, of course, that there are certain deficiencies in attempting to define the charge capacity of microcarriers strictly on a unit weight basis. For example, two beads identical in every way except that they are formed from materials having different densities with the same charge distribution thereon would yield different values for their charge capacity per unit weight. Similarly, two beads having identical charge capacities per unit weight might have quite different charge distributions thereon.

An alternative definition can be made by specifying the range of suitable charges in terms of charge capacity per unit weight of microcarriers in their final functional form. This basis would take into account such factors as the weight of attached DEAE or other positively charged groups, as well as hydration of the beads, etc., whereas the prior definition is based on dry, crosslinked dextran and does not take such factors into account. In an aqueous cell culture medium, the density of microcarriers should be close to 0.1 gram/cc so that the microcarriers can be readily dispersed throughout the culture. Based upon this, it has been determined that the range of suitable charge capacities for microcarriers of this invention defined in this way is from about 0.012 to about 0.25 meq/gram.

The ranges of suitable charge capacities previously specified on a weight basis are valid assuming the microcarriers have a substantially uniform charge distribution throughout their bulk. If the charge distribution is uneven, it might be possible to have suitable microcarriers having charge capacities outside of those ranges. The important criterion is, of course, that the charge capacity be adjusted to and/or controlled at a value sufficient to allow good cell growth on the microcarriers.

Since it may be the charge pattern on the outer surface which is important, it is also desirable to be able to define a suitable charge capacity range in terms of the likely surface pattern. This can be done by assuming that the active portion of the microcarriers represents only the outer surface of the bead to a depth of about 20 angstroms. If it is also assumed that the charged groups in the previously mentioned cases are evenly distributed throughout the beads, the previous ranges can be converted to a charge capacity in this outer shell. Using this approach, the range of charge capacity found suitable is from about 0.012 meq/cm^3 to about 0.25 meq/cm^3. This approach takes changes in microcarrier volume due to different charge densities into account.

Microcarriers having the required charge capacity can be prepared by treating microcarriers formed from polymers containing pendant hydroxyl groups with an aqueous solution of an alkaline material and a tertiary or quaternary amine. The beads can be initially swollen in an aqueous medium without the other ingredients, or can be simply contacted with an aqueous medium containing the required base and amine. This method of using alkaline materials to catalyze the attachment of positively charged amino groups to hydroxyl-containing polymers is described in Hartmann, U.S. Pat. No. 1,777,970.

Examples of suitable hydroxyl-containing polymers include polysaccharides such as dextran, dextrin, starch, cellulose, polyglucose and substituted derivatives of these. Certain synthetic polymers such as polyvinyl alcohol and hydroxy-substituted acrylates or methacrylates, such as hydroxyethyl methacrylate, are also suitable. Dextran, and especially crosslinked dextran in the form of small spheres or beads, is particularly preferred because it is commercially available, relatively inexpensive, and produces microcarriers which support excellent cell growth.

Any material which is alkaline can be used for the reaction. The alkali metal hydroxides, such as sodium or potassium hydroxide, are, however, the preferred alkaline substances.

Either tertiary or quaternary amines are suitable sources of positively charged groups which can be appended onto the hydroxy-containing polymers. Particularly preferred materials are chloro- or bromo-substituted tertiary amines or salts thereof, such as diethylaminoethylchloride, diethylaminoethylbromide, dimethylaminoethylchloride, dimethylaminoethylbromide, diethylaminomethylchloride, diethylaminomethylbromide, di-(hydroxyethyl)-aminoethylchloride, di-(hydroxyethyl)-aminoethylbromide, di-(hydroxyethyl)-aminomethylchloride, di-(hydroxyethyl)-aminomethylbromide, β-morfolinoethylethylchloride, β-morfolinoethylbromide, β-morfolinomethylchloride, βmorfolinomethylbromide and salts thereof, for example, the hydrochlorides.

A wide range of reaction temperatures and times may be used. It is preferred to carry out the reactions at temperatures of about between 18° C. and 65° C. However, other temperatures can be used. The reaction kinetics depend to a large extent, of course, upon the reaction temperature and the concentration of reactants. Both the time and temperature do affect the final exchange capacity achieved.

The reason that the charge capacity of the microcarriers is so critical in cell growth is not thoroughly understood. While not wishing to be bound by this theory, it is possible that the charge capacity at the surface causes certain local discontinuities of medium composition which are the major controlling influence in microcarrier culture cell growth. Nevertheless, this is not meant to rule out other possibilities.

There may be certain beads, of course, that will not be suitable for good cell growth even though they have a charge capacity within one of the ranges specified. This may be due to side chains on the moiety supplying the charge capacity which are toxic or otherwise deleterious for cell growth, the presence of adsorbed or absorbed deleterious compositions or compounds, or it may be due to the porosity of the bead or due to other reasons. If such beads are not suitable for cell growth except for the amount of charge capacity, the beads are not considered to be "cell-growth microcarriers."

The invention is further illustrated by the following examples.

EXAMPLE I

Preparation of Improved Microcarriers

Improved microcarriers can be produced as follows. Dry, uncharged, crosslinked dextran beads are seived to obtain those of approximately 75 μm in diameter. One

droxide in 20 ml of distilled water. The solution was then poured over dry Sephadex G-50 beads after which the beads were placed on a reciprocating shaker-water table maintained at 60° C. One set of bead batches was treated with a solution containing 0.01 moles of the amine and 0.015 moles of sodium hydroxide, whereas another set of batches was treated with a solution containing 0.03 moles of the amine and 0.045 moles of sodium hydroxide. The reaction time was varied to produce different meq/g within each batch.

Diploid human fibroblasts (HEL299) were grown in suspension cultures at a microcarrier concentration of 5.0 grams dry, untreated crosslinked dextran per liter following the procedures of Example 2 using microcarriers having varying meq/gram selected from each batch. Subsequently, productivity (10^6 cells grown/liter hour) was calculated and plotted versus meq/gram for each batch of beads produced as above. Curves plotted using data obtained for both sets were similar in shape, having a general bell shape, but the curve from the batches treated with the higher concentration of reactants had a somewhat sharper rise and fall. Carriers yielding excellent cell growth were produced from each batch.

EXAMPLE 4

Preparation of Microcarriers at Varying Amine/Alkali Ratios

This example illustrates further changes in the charge capacity which can be obtained by varying DEAE chloride:chloride/NaOH ratios. In this example, the procedures of Example 3 were followed except that a wide range of concentrations of sodium hydroxide was used while maintaining the concentration of the diethylaminoethylchloride:chloride at 0.01 moles per 20 ml. The concentrations used for the sodium hydroxide were 0.01, 0.011, 0.012, 0.013, 0.014, 0.015, 0.02, 0.03, 0.05, 0.75, 0.10 moles per 20 ml.

A plot was made of meq/gram after 1.25 hours at 60° C. versus concentration of sodium hydroxide. It was observed from the plot that concentrations of sodium hydroxide below about 0.01 produced no detectable charge capacity. Charge capacity rose quickly, however, with increases in concentration and reached a maximum of around 2.3 meq/gram dry, crosslinked dextran at a concentration of about 0.014 moles sodium hydroxide. Charge capacity then declined in an almost linear relationship to a value of about 1. meq/gram at a sodium hydroxide concentration of about 0.10 moles. Thus, a change in reaction kinetics takes place when the ratio of DEAE Chloride: chloride to sodium hydroxide is varied at a constant concentration of DEAE chloride:chloride and crosslinked dextran.

EXAMPLE 5

Human Interferon Production in Cells Grown on Improved Microcarriers

The ability of microcarrier grown cells to produce human interferon is described herein. Cells used for the production of human interferon were normal diploid human foreskin fibroblasts, FS-4. These fibroblasts were grown in microcarrier cultures using procedures as in Example 2. Microcarriers prepared and titrated according to Example 1 were used at a concentration of 5 grams of dry, crosslinked dextran/liter. The medium used for culture growth was DMEM supplemented with 10% fetal calf serum.

In 8 to 10 days, cultures ceased growing. At this point, growth medium was removed. Cultures were washed 1–4 times with 100 ml of serum-free DMEM. The cells were then ready for interferon induction. This was accomplished by adding to the cultures 50 ml of serum-free DMEM medium containing 50 μg/ml cyclohexamide, and varying amounts of poly I poly C inducer. After 4 hours, Actinomycin D was added to the cultures to a final concentration of 1 μg/ml.

Five hours after the onset of induction, inducing medium was decanted and cultures were washed 3–4 times with 100 ml of warm serum-free DMEM. Cultures were replenished with 50 ml of DMEM containing 0.5% human plasma protein. Cultures were incubated under standard conditions for an additional 18 hours. At this time, cultures were decanted, and the decanted medium was assayed for interferon activity. Interferon activity was assayed by determining the 50% level of cell protection for samples and standard solutions, for FS-4 fibroblasts challenged with Vesicular Stomatitis Virus (VSV), Indiana strain. The results of interferon production runs are presented in tabular form below.

Inducer Concentration (μg/ml)	Cell Concentration During Production (cells/ml)	Interferon (U/10^6 cells)
4	2.0×10^6	39
5	2.6×10^6	378
25	2.6×10^6	886
50	2.0×10^6	~5000

These data are each from a separate run and are not intended to demonstrate any correlation to inducer concentration.

EXAMPLE 6

Growth of Cells on Improved Microcarriers for the Purpose of Producing Viruses

The ability of microcarrier grown cells to produce a virus is described here. Primary and secondary chicken embryo fibroblasts were grown in microcarrier culture according to the procedure described in Example 2 with the primary cells grown at 10 grams/liter and the secondary at 5 grams/liter microcarrier concentration. To initiate virus production, growth medium was removed, and the cultures were washed twice with 100 ml of serum free DMEM. Infection of cells with Sindbis virus took place in 50 ml of DMEM supplemented with 1% calf serum, 2% tryptose phosphate broth, and enough Sindbis virus to equal an MOI (multiplicity of infection) of 0.05.

The virus was harvested 24 hours after infection, by collecting culture broth, clarifying at low centrifugation, and freezing the supernatant. Virus production was assayed by plaque formation in a field of secondary chicken fibroblasts. The results of infecting these microcarrier cultures were:

Cell Type	All Concentration For Production (cells/ml)	(PFU/ml)	PFU/cell
Secondary	4.0×10^6	8.4×10^9	2,100
Primary	1.4×10^6	2.3×10^{10}	16,000
Primary	6.0×10^6	2.6×10^{10}	5,000

Virus production was also established for the following virus/cell on microcarrier combinations: Polio/WI-38; Moloney MuLV/Cl-1 mouse and VSV/chick embryo fibroblasts.

EXAMPLE 7

Comparative Growth of Cells in Roller Bottles and with Improved Microcarriers for the Purpose of Producing Murine Leukemia Virus Proviral DNA

The reverse-transcribed DNA of Moloney leukemia virus (M-MuLV) after infection of JLS-V9 cells, a mouse bone marrow line, was studied.

One technique involved growing cells in roller bottles. Cells were grown in roller bottle culture, the medium removed, and virus inoculum introduced into the bottles. Shortly thereafter, the cultures were fed with fresh medium, and 8–16 hours later extracted for eventual purification of viral DNA. The cultures were washed with fresh buffer and the cell lysed with a solution containing the detergent sodium dodecylsulfate. Subsequent cooling of the lysate and addition of salt to one molar caused co-precipitation of the detergent with high molecular weight DNA. The low molecular weight DNA remaining in the supernatant could then be deproteinized and concentrated for further analysis.

A 50-roller bottle culture contained about 10^9 cells. These were infected with about one-liter of viral inoculum titering at 3×10^6 plaque-forming units per ml. This resulted in a nominal multiplicity of infection of 1–3 and the infected cells yielded 5–20 nanograms of virus-specific DNA.

A simpler procedure was developed employing improved microcarriers according to this invention. A culture containing 10 grams of beads in one liter of growth medium was used. Upon reaching confluence, the 10^9 cells on the beads were infected by allowing the beads to settle out and replacing the medium with 1 liter of virus inoculum. For extraction, the cells on the beads were washed with buffer and then placed in the SDS containing buffer. After co-precipitation of the high molecular weight DNA with the detergent, the precipitate together with the beads were centrifuged out and a supernatant extracted for further analysis. The yield of viral DNA was comparable to that obtained in roller bottle culture and the labor involved was 5–10% of that required by roller bottle culture.

EXAMPLE 8

Improved Microcarrier Production with Dimethylaminoethyl Charge Groups

A suitable microcarrier was produced by binding an alternate exchange moiety to the dextran matrix utilized in Example 1. Dimethylaminoethyl groups (DMAE) were bound to a dextran matrix by the following procedure: 1 gm of dextran beads (Pharmacia G-50), 50–75 μm in diameter, dry, was added to 10 ml of distilled water and the beads were allowed to swell. An aqueous solution containing 0.01 moles of dimethylaminoethylchloride:chloride (Sigma Chemical Co.) and 0.015 moles of sodium hydroxide was formed in a 10 ml volume. This aqueous solution was added to the swollen dextran beads and this suspension was then agitated vigorously for one hour at 60° C. After reaction, the bead mass was titrated as in Example 1. This reaction binds 1.0 meq of dimethylaminoethyl to the dextran mass. To produce microcarriers of greater degrees of substitution, the above reaction was carried out, and the bead mass washed thoroughly with water. With excess water filtered off, the bead mass was weighed so as to determine the amount of water being retained by the bead mass. To this bead mass was added the appropriate amount of fresh reagents (i.e., DMAE-CL:CL, and NaOH) so that the final concentration of DMAE, and NaOH in these succeeding reaction mixtures were identical to those initially used.

In this manner, a series of microcarriers were prepared at 1.0, 2.0, 2.5 and 3.5 meq DMAE/gm unreacted dextran. Cells (HEL 299) were grown in microcarrier culture (5 gm/l) with these microcarriers according to the procedures in Example 2. The results are tabulated in the following table:

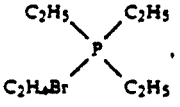
Degree of Substitution (meq/gm)	Cell Spreading	Net Growth
1.0	−	−
2.0	−	−
2.5	+	+
3.2	+	−

As expected, cell growth is related to the degree of substitution with charge carrying groups. At too high a degree of substitution, no cell growth occurs, although attachment and spreading takes place. At too low a degree of substitution, cell adhesion to the surface is not sufficient to allow proper spreading and growth.

EXAMPLE 9

Improved Microcarriers Having Positively Charged Phosphonium Groups

Improved microcarriers were also prepared using non-amine exchange groups as follows. One gram of dry dextran beads were prepared and swollen with water as in Example 1. To the swollen beads were added 5 ml of a saturated aqueous solution of triethyl-(ethyl-bromide)-phosphonium (TEP),



and 5 ml of a 3 molar solution of sodium hydroxide. This slurry was reacted at 65° C. A series of microcarriers were prepared at 1.1, 1.7 and 2.9 meq/gm. The microcarriers at 1.1 meq/gm were prepared by reaction at the above conditions for 4 minutes. The 1.7 meq/gm microcarrier was reacted for 1 hour, and the 2.9 meq/gm microcarriers were reacted successively 3 times as described in Example 7. A microcarrier cell culture at 5 gm/liter was established for each of these carriers with a continuous cell type, JLS-V9 and compared to this cell's growth on improved DEAE-microcarriers prepared as in Example 3. The results are tabulated in the following table.

meq/gram	Cell Attachment and Spreading	Net Growth
	DEAE	
0.9	+	+
1.7	+	+
3.8	+	−
	TEP	
1.1	+	+

-continued

meq/gram	Cell Attachment and Spreading	Net Growth
1.7	+	+
2.9	+	-

It will be recognized by those skilled in the art that there are certain equivalents to the specific techniques, materials, etc., described herein, and these are considered to be part of this invention and are intended to be covered by the following claims. Additionally, while most of the description herein has been limited to the use of the improved microcarriers for growth of anchorage-dependent cells, they can also be used, of course, for the growth of other cell types.

What is claimed is:

1. Cell culture microcarriers having a degree of substitution thereon with positively-charged chemical moieties sufficient to provide a charge capacity of from about 0.1 to about 4.5 meq/gram of dry, untreated microcarriers.

2. Cell culture microcarriers comprising crosslinked dextran beads having a sufficient amount of positively charged groups thereon to provide a charge capacity of between about 0.1 and about 4.5 meq/gram of dry, crosslinked dextran beads.

3. Cell culture microcarriers of claim 2 wherein said positively charged groups comprise diethylaminoethyl groups.

4. Cell culture microcarriers comprising a reaction product of crosslinked dextran beads and an aqueous solution of a tertiary or quaternary amine and a base, said aqueous solution having an amount and ratio of amine and base sufficient to provide said microcarriers with an exchange capacity of from about 0.1 to about 4.5 meq/gram of dry dextran.

5. Cell culture microcarriers of claim 4 wherein said amine comprises diethylaminoethyl.

6. Cell culture microcarriers of claim 5 wherein said base comprises sodium hydroxide.

7. A method of producing cell culture microcarriers comprising soaking crosslinked dextran beads in an aqueous solution of a tertiary or quaternary amine and a base until said beads are substituted with a sufficient amount of amine moieties to produce an exchange capacity thereon of from about 0.1 to about 4.5 meq/gram of dry dextran.

8. A method of claim 7 wherein said amine comprises diethylaminoethyl.

9. A method of claim 8 wherein said base comprises sodium hydroxide.

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