Principles and methods

Microcarrier cell culture

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Contents

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01
Introduction

The pressure in biotechnology production today is for greater speed, lower costs and more flexibility. Ideally, a production unit should be compact (requires less investment) and modular (for use in different production schemes) (Fig 1). Designs that allow the same plant to be used for different cell types (bacteria, yeast, insect cells, plus suspension and anchorage-dependent animal cells) are thus preferable. Animal cell culture is constantly being developed to increase unit productivity and thus make the production costs of animal cell products more competitive.

Because animal cells have a relatively low productivity, large amounts of culture supernatant are needed per clinical dose of final product. Extremely large volume cultures will be needed to produce kilogram quantities of a therapeutic monoclonal antibody, for example.
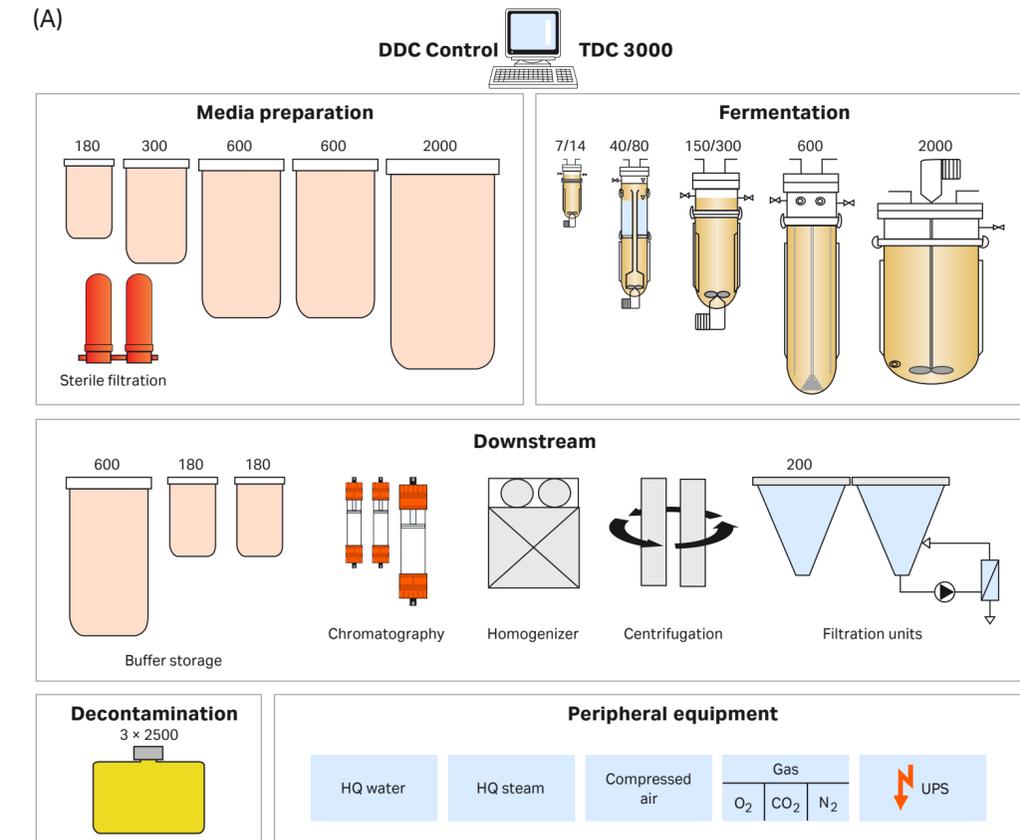
Fig 1A. Scheme of a production plant.
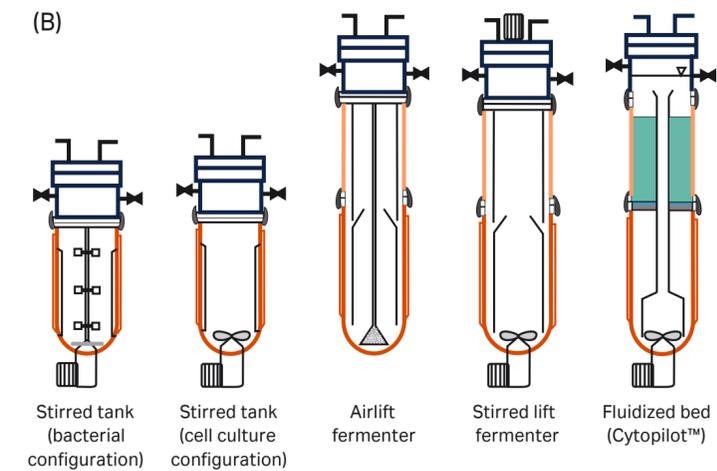
Fig 1B. Different reactor configurations. Courtesy of Polymun Scientific GmbH, Vienna, Austria.

Animal cell culture is currently operated at 15 000 liter scale in suspension cultures and 6000 liter scale in microcarrier cultures of anchorage-dependent cells (Fig 2). Both are large unit operations. As the size of such operations increases, so does investment in resources and personnel. Cell culture conditions are also more critical. Failures become far more costly.

The productivity of large-scale cell culture can be increased either by scaling up to larger volumes with cell densities of $2–3 \times 10^6$/mL, or by intensifying the process in smaller volumes but with higher cell densities (up to 2×10^8 cells/mL). When intensifying cell densities, more frequent media changes are needed and eventually perfusion has to be applied. Many competing technologies are available.

Microcarrier culture of anchorage-dependent or entrapped cells lowers the volume to cell density ratio and thus belongs to the second of the above alternatives. This technique has many advantages for the commercial manufacturer. It operates in batch or perfusion modes and is well suited to efficient process development and smooth scale-up (Fig 3). In addition, the reactors can be modified to grow other organisms.

This handbook reviews the principles and methods of using microcarriers in cell culture production.

Fig 3A. Different microcarriers and applications.
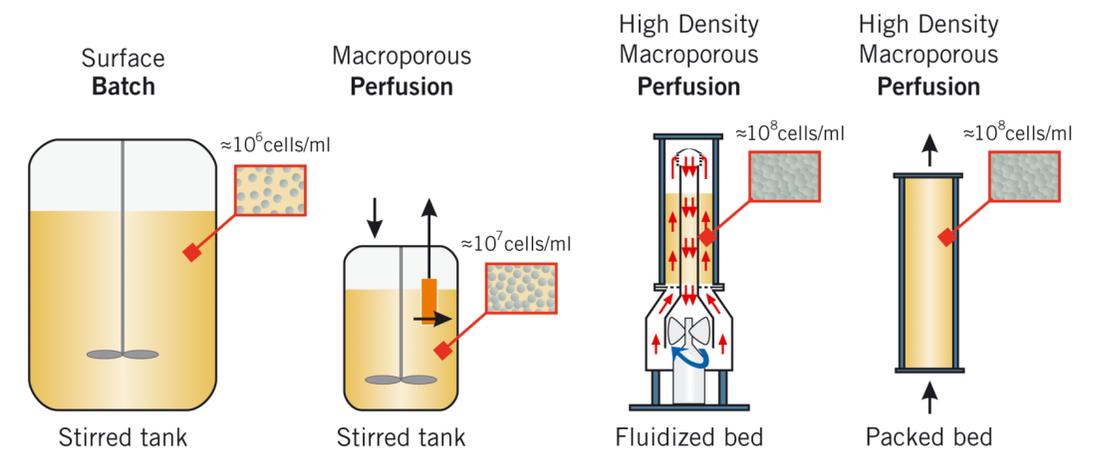
Fig 3B. Different microcarrier culture setups.

Fig 2. Influenza production plant (6000 liter vessel for cultivating Vero cells on Cytodex™). Courtesy of Baxter Biosciences.

1.1 Reasons for using microcarriers

Cell culture techniques have become vital to the study of animal cell structure, function and differentiation, and for the production of many important biological materials such as vaccines, enzymes, hormones, antibodies, interferons and nucleic acids. Microcarrier culture introduces new possibilities and, for the first time, makes possible the practical high-yield culture of anchorage-dependent cells. In microcarrier culture, cells grow as monolayers on the surface of small spheres (Fig 4) or as multilayers in the pores of macroporous structures that are usually suspended in culture medium by gentle stirring. By using microcarriers in simple suspension culture, fluidized or packed bed systems, yields of up to 200 million cells per milliliter are possible.

Cytodex microcarriers have been specifically developed by Amersham Biosciences (now a part of Cytiva) for the high-yield culture of a wide range of animal cells (Section 5.1.3) in culture volumes ranging from a few milliliters to several thousand liters. The special requirements of the microcarrier system are best fulfilled by dextran-based beads that are subsequently derivatized to form two types of Cytodex, cellulose-based beads to form two types of Cytopore™.
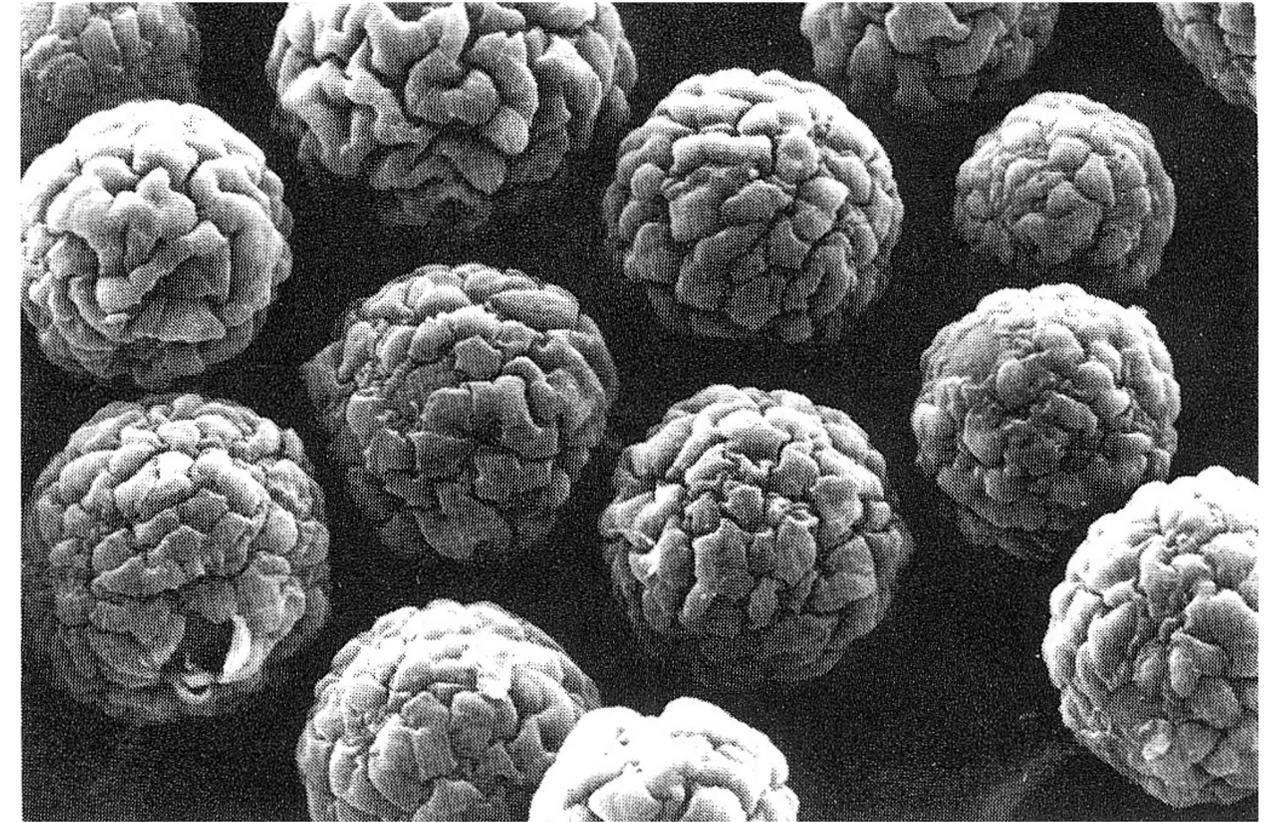
Fig 4. Scanning electron micrograph of pig kidney cells (IBR-S2) growing on Cytodex 72 h after inoculation. (Original photograph by G. Charlier, INVR, Brussels, Belgium, reproduced by kind permission.)

Cytodex:

- Surface characteristics have been optimized for efficient attachment and spreading of cells

- Size and density are optimized to facilitate even suspension and give good growth and high yields for a wide variety of cells

- Matrix is biologically inert and provides a strong but non-rigid substrate for stirred cultures

- Transparent and allows easy microscopic examination of the attached cells

Experience with Cytodex in a wide variety of applications has confirmed its importance and value in microcarrier techniques.

Cytopore:

- Cytopore, a macroporous microcarrier, is a logical development of Cytodex that increases cell density (and therefore yield) and allows suspension cell cultivation and perfusion

1.1.1 New opportunities and applications for animal cell culture

Microcarriers provide convenient surfaces for growing animal cells or increasing the yield of cells from standard monolayer culture vessels and perfusion chambers. Applications include production of large quantities of cells, viruses and cell products, studies on differentiation and cell function, perfusion culture systems, microscopy studies, harvesting mitotic cells, isolation of cells, membrane studies, storage and transportation of cells, assays involving cell transfer and studies on uptake of labeled compounds (see Section 4 for a description of these applications).

1.1.2 Increased production capacity

The very large culture surface area to volume ratio offered by the microcarrier system (e.g., 30 cm^2 in 1 mL using 5 mg Cytodex 1) provides high cell yields without having to resort to bulky equipment and tedious methodology. For a given quantity of cells or their products, microcarrier cultures demand much less space (Fig 40) than other types of monolayer culture. Culturing cells in small compact systems is especially important when working with pathogenic organisms.

1.1.3 Separating cells from secreted product

Microcarrier technology is one method for cell retention (Fig 5), which means that dilution rate (perfusion rate) becomes independent of the growth rate of the cells. Secondly, because there are fewer cells in the harvest stream, downstream processing becomes simpler. You transfer part of the clarification step back into the fermentation process.
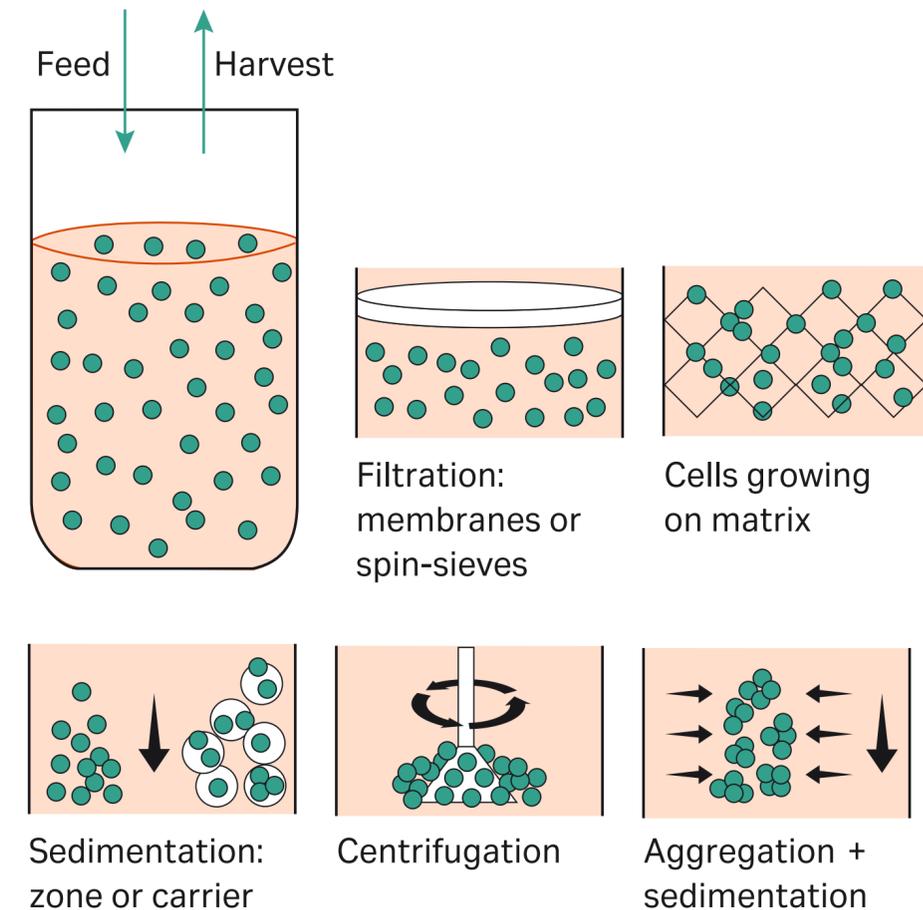
Fig 5. Different retention systems.

1.1.4 Improved control

Microcarrier systems allow excellent control of culture parameters (e.g., pH, gas tensions, etc). The technique provides a method for growing anchorage-dependent cells in a system having all the advantages of suspension culture. Improved control allows homogeneous culture systems with a wide variety of process designs (1). Monitoring and sampling microcarrier cultures is simpler than with any other technique for producing large numbers of anchorage-dependent cells.

1.1.5 Protection against physical and chemical stress

Macroporous microcarriers protect cells against the tip of the stirrer, especially at large scale. Sparging with microbubbles using pure oxygen is also possible due to this protection. Cells tolerate more chemical stress such as lactate, ammonia and oxygen if they create a micro-environment inside the pores (Fig 6).

1.1.6 Reduced requirements for culture medium

When compared with other monolayer or suspension techniques, stirred microcarrier cultures yield up to 100-fold as many cells for a given volume of medium. Superior yields have been reported for a wide variety of systems including chicken fibroblasts (2, 3), pig kidney cells (4), fish cells (5), Chinese hamster ovary cells (6), human fibroblasts (7), primary monkey kidney cells (8) and transformed mouse fibroblasts (9). This reduced requirement for medium means considerable savings in cell culture costs (6, 9), particularly when expensive serum supplements such as fetal calf serum are used. Macroporous microcarriers especially provide an excellent micro-environment where cells can exchange their own autocrine growth hormones, thereby allowing protein-free media to be used.

1.1.7 Reduced requirements for labor

Because large numbers of cells can be cultured in small volumes (more than 10^{11} cells/L), fewer culture vessels are required with microcarrier cultures. For example, one technician can handle a vaccine production equivalent to 900 roller bottles per week (10). One liter of microcarrier culture can yield as many cells as up to 50 roller bottles (490 cm^2 bottles, 2), and 1 to 1.5 mL of macroporous beads can yield as many cells as one roller bottle of 850 cm^2. The simplified procedures associated with microcarriers reduce the labor needed for routine production. Separating cells from the culture medium is simple; when stirring stops, microcarriers with cells attached settle under the influence of gravity and the supernatant can be removed. Unlike true suspension cell culture systems, no centrifugation steps are necessary.

1.1.8 Lower risk of contamination

In cell culture, the risk of contamination is related to the number of handling steps (e.g., opening and closing culture vessels) required to produce a given quantity of cells or products. Microcarrier culture reduces the number of handling steps. There is a much-reduced risk of contamination when producing a large quantity of cells in a single microcarrier culture than in several hundred roller bottles (6).
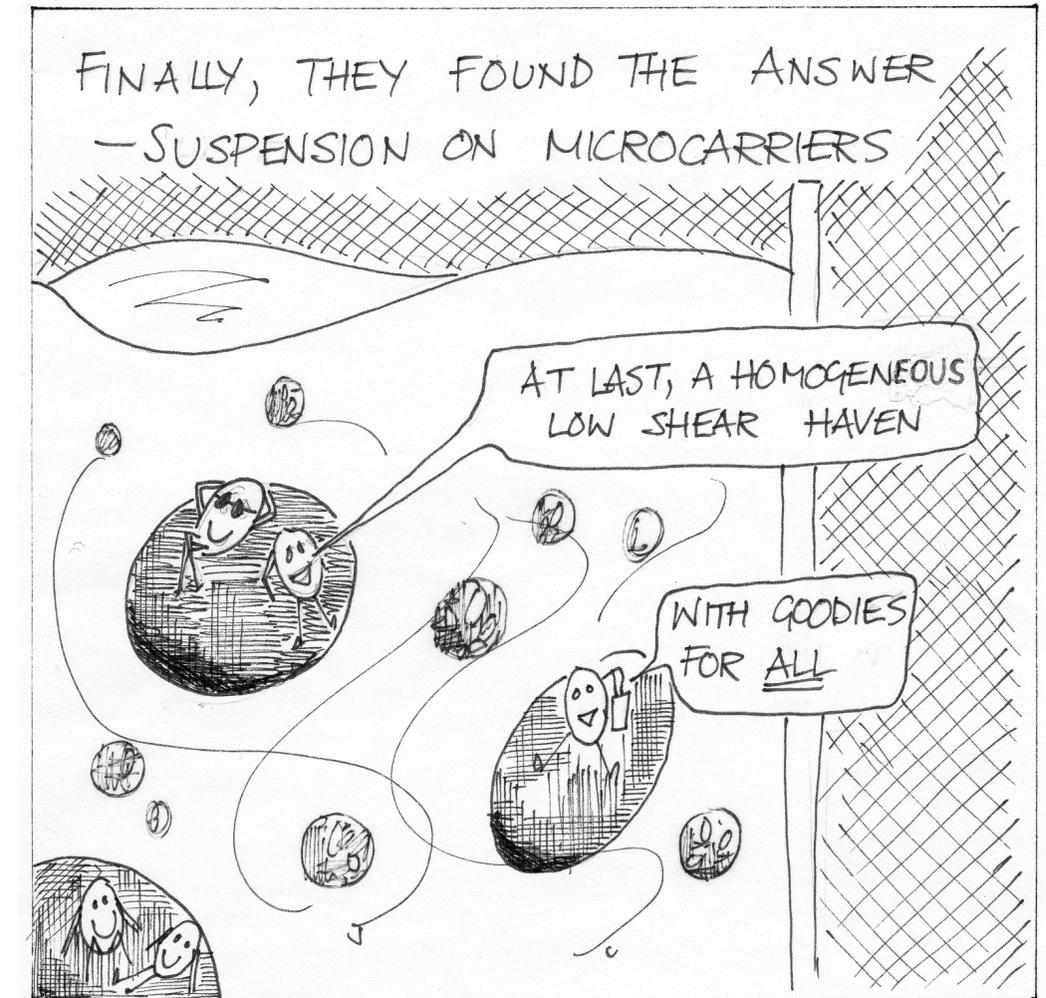
Fig 6. Graph of cells sitting satisfied inside pores.

1.2 Aim of this handbook

This handbook describes the principles and methods of achieving the best cell culture results with Cytodex and Cytopore microcarriers. Although this technique is one of the most advanced in animal cell culture, it need not be restricted to experienced cell culturists. Because cell culture is used by a wide variety of scientists, this handbook is written for both beginners and those more experienced in cell culture. Only a basic knowledge of cell culture is assumed.

This handbook aims to help the reader plan and operate optimum procedures with the minimum of effort, and to achieve consistent results with high yields. All methods have been developed for Cytodex and Cytopore and are not necessarily suitable for other cell culture surfaces.

02
Microcarrier background

2.1 Adhesion (cell-cell, cell-surface)

Cell-cell and cell-surface (matrix) adhesion is one of the most important processes in higher life forms. This mechanism allows specialized cell conglomerates that result in different tissues, organs and skeletal structures. In vertebrates, the major types of tissue are nerve, muscle, blood, lymphoid, epithelial, and connective tissue.

In principle, there are three functional classifications of junctions between cells: occluding junctions, communicating junctions and anchoring junctions. Occluding or tight junctions can seal cells together in an epithelial cell sheet in a way that prevents even small molecules from leaking from one side to the other. Communicating junctions mediate the passage of chemical or electrical signals from one interacting cell to its partner. Anchoring junctions, the most interesting ones for this work, mechanically attach cells (and their cytoskeletons) to their neighbors or to extracellular matrices. Both kinds of attachment are interesting for animal cell culture methods, as artificial matrices may simulate or stimulate cell-cell or cell-matrix junctions.

In vivo, many different molecules are involved in attachment processes that are localized intracellularly, extracellularly and/or in the cell membrane.

Intracellular attachment proteins (Table 1) connect the cytoskeleton with transmembrane linker proteins, which bind to transmembrane linker proteins of other cells in a homophilic or heterophilic binding. Extracellular linker molecules also mediate the binding of transmembrane linker proteins. Such connections of the cytoskeletal actin filament and transmembrane linker proteins (Ca^{2+}-dependent cadherins), which are mediated by catenins, are called anchoring junctions.

Table 1. Cell adhesion molecule families

Adhesion	Protein groups	Ca^{2+} or Mg^{2+} dependency	Homophilic/ heterophilic	Cytoskeleton association	Cell junction association
Cell-cell	cadherins	yes	homophilic	actin filaments	adhesion belt
Cell-cell	Ig family (N-CAM)	no	homophilic/ heterophilic	unknown	no
Cell-cell	selectins	yes	heterophilic	unknown	no
Cell-cell	integrins	yes	heterophilic	actin filaments	no
Cell-matrix	integrins	yes	heterophilic	actin filaments (via talin, vinculin and other proteins)	focal contacts/ hemidesmosomes
Cell-matrix	trandmembrane proteoglycans	no	heterophilic	actin filaments	no

There are four different classes of anchoring junctions (Table 2): 1, adherence junctions (cell-cell); 2, adherence junctions (cell-matrix); 3, desmosomes; and 4, hemidesmosomes. Adherence junctions connect bundles of actin filaments cell-to-cell or cell-to-extracellular matrix. Desmosomes connect intermediate filaments cell-to-cell, and hemidesmosomes connect the intermediate filaments to the basal lamina (a specialized mat of extracellular matrix at the interface between the epithelium and connective tissue).

Table 2. Anchoring junctions

Junction	Transmembrane linker protein	Extracellular ligand	Intracellular cytoskeletal attachment	Some intracellular attachment proteins
Adherence (cell-cell)	cadherin (E-cadherin)	cadherin in neighboring cell	actin filaments	catenins, vinculin, α-actinin, plakoglobin
Adherence (cell-matrix)	integrin	extracellular matrix protein	actin filaments	talin, vinculin, α-actinin
Desmosome	cadherin	cadherin in neighboring cell	intermediate filaments	plakoglobin
Hemidesmosome	integrin	extracellular matrix (basal lamina) proteins	intermediate filaments	desmoplakinlike protein

In epithelial sheets, anchoring junctions often form a continuous adhesion belt (*zonula adherens*). The actin filaments are further connected to a set of intracellular attachment proteins that include α, β and γ-catenin, vinculin, α-actinin, and plakoglobin. The actin bundles are linked in an extensive intracellular network that can be contracted by myosin motor proteins to mediate a fundamental process in morphogenesis, which is important for the activity of the cells. In addition, a family of cell-surface carbohydrate-binding proteins (lectins) called selectins play a role in a variety of transient cell-cell adhesion interactions in the bloodstream. Selectins contain a highly conserved lectin domain that, in the presence of Ca^{2+}, binds to a specific oligosaccharide on the surface of other cells, resulting in a heterophilic cell-cell adhesion.

Ca^{2+}-independent cell-cell adhesion is mediated mainly by members of the immunoglobulin superfamily of proteins, such as the neural cell adhesion molecules (N-CAM) that are involved in homophilic interactions. Heterophilic interactions are mediated by intercellular adhesion molecules (ICAMs), which interact with integrins present on the surface of white blood cells, thereby helping to trap these blood cells at sites of inflammation.

There are two main classes of extracellular macromolecules that make up the matrix in vertebrates:

• Polysaccharide chains of the class called glycosaminoglycans (GAG), which are usually found covalently linked to protein in the form of proteoglycans

• Fibrous proteins of two functional types: mainly structural (for example, collagen and elastin) and mainly adhesive (for example, fibronectin and laminin)

Members of both classes vary greatly in shape and size.

Glycosaminoglycans (GAGs) consist of unbranched polysaccharide chains composed of repeated disaccharide units, where one of the two sugar residues is always an amino sugar (N-acetylgycosamine or N-acetylgalactosamine), which in most cases is sulfated. The second sugar is usually uronic acid (glucuronic or iduronic). Because there are sulfate or carboxyl groups on most of the sugar residues, GAGs are highly negatively charged. Four main groups of GAGs have been distinguished by their sugar residues according to the type of linkage between these residues and the number and location of sulfate groups: 1st hyaluronan, 2nd chondroitin sulfate and dermatan sulfate, 3rd heparan sulfate and heparin and 4th keratan sulfate.

GAGs and proteoglycans are capable of self-organizing into large complexes that have the size of bacteria. Moreover, besides associating with one another, GAGs and proteoglycans associate with fibrous matrix proteins such as collagen, and with protein meshworks such as the basal lamina, creating extremely complex structures. Furthermore, they have different functions *in vivo*. Due to their inflexible, strongly hydrophilic nature, GAGs occupy a huge volume relative to their mass, and they form gels even at very low concentrations. Their high density of negative charges attracts a cloud of cations, such as Na^+, causing large amounts of water to be sucked into the matrix, resulting in a swelling pressure, or turgor, that enables the matrix to withstand compressive forces (in contrast to collagen fibrils, which resist stretching forces).

Proteoglycans can also regulate the activities of secreted signaling molecules, such as enhancing or inhibiting the activity of growth factors. Furthermore, proteoglycans also bind and regulate the activities of other types of secreted proteins in any of the following ways: 1, they can immobilize the protein at the site where it is produced, thereby restricting its range of action; 2, they can sterically block the activity of the protein; 3, they can provide a reservoir of the protein for delayed release; 4, they can protect the protein from proteolytic degradation, thereby prolonging its action; and 5, they can alter or concentrate the protein for more effective presentation to cell-surface receptors.

A fibrous group of proteins, the collagens, belongs to the second group of extracellular macromolecules. These are the major proteins of the extracellular matrix *in vivo*, thus cells normally attach tightly to these structures. This attachment is mediated by the second group of proteins in the matrix, the adhesive proteins such as fibronectin. Fibronectin contributes to both organizing the matrix and helping cells attach to it. It forms dimers composed of very large subunits joined by a pair of disulfide bonds near their carboxyl termini. Each subunit is folded into a series of functionally distinct rod-like domains separated by regions of flexible polypeptide chains. These domains consist of smaller modules, each of which is serially repeated. These different domains bind to collagen, heparin, and specific receptors on the surface of various types of cells. The sequence responsible for cell binding is a specific tripeptide (Arg-Gly-Asp, or RGD). Even very short peptides containing this RGD sequence compete with fibronectin for the binding sites on cells. Each receptor, however, specifically recognizes its own small set of matrix molecules, indicating that tight receptor binding requires more than just an RGD motif.

The last class of important proteins is the integrins. These are the principal receptors used by animal cells to bind to the extracellular matrix. They are heterodimers that function as transmembrane linkers to mediate bidirectional interactions between the extracellular matrix and the actin cytoskeleton. They also function as signal transducers, activating various intracellular signaling pathways when activated by matrix binding. A cell can regulate the adhesive activity of its integrins by altering either their matrix-binding sites or their attachment to actin filaments.

Integrins located in the plasma membrane anchor a cell to extracellular matrix molecules; cadherins in the plasma membrane anchor the cell to cadherins in the membrane of an adjacent cell. In both cases, there is an intracellular coupling to cytoskeletal filaments, which can be either actin or intermediate filaments depending on the types of intracellular attachment proteins employed. Moreover, for all these classes of anchoring junctions, the adhesion depends on extracellular divalent cations, although the significance of this dependence is unknown.

Cell adhesion is a multistep process involving initial contact of the cell with the surface, cell spreading on the surface, and cell differentiation or growth. Attachment involves forming bonds between transmembrane adhesion receptors (e.g., cadherins, integrins and N-CAMs) and absorbed adhesion proteins (e.g., fibronectin, vitronectin, laminin). Binding induces a conformational change in the cytoplasmic domain of the receptor, which produces receptor aggregation (11). Free receptors diffuse into the contact area forming more interactions with adhesion proteins and increasing the size of the contact area (12). Cell spreading involves interaction among microtubules, actin filaments, and ligand-bound adhesion receptors (13). Cell spreading is the critical step in adhesion and subsequent cell growth and protein formation (14,15).

Anchorage-dependent cells need a certain amount of the described attachment proteins to proliferate *in vitro*. This requirement can be achieved by adding serum to the media or by coating the growth surfaces with collagen or other attachment proteins, such as fibronectin or variants of the cell recognition site of fibronectin (RGD, RGDS, RGDV, RGDT, and other peptides) (17–20). However, these substances often derive from animal products, and are therefore considered to be of potential risk for biological safety. A solution to this problem is the use of recombinant proteins produced in prokaryotic or lower eukaryotic cells. However, disadvantages of this method are the high price of such products, and the fact that media for bacteria or yeast may contain some sort of animal-derived hydrolysates.

Plant-derived biomolecules are therefore better suited to increase the adherence of production cell lines. As such plant-derived macromolecules do not enhance adherence per se (as shown below), chemical derivatization is a useful tool to engineer biomimetic materials. For this purpose, the theory of Emil Fischer (1852–1919) — the proposed key-lock system — might provide a possible method. Based on knowledge of cell adherence *in vivo* (see above), artificial substances with the required characteristics can be designed. Polymers can be used as a string to which keys are attached (21). Random substitution of the macromolecular chains allows the formation of specific binding sites in a composition-dependent way. The polymer is used as a kind of master key with an infinite amount of different specific keys attached to it. These keys need a recognition site, so that the water can be displaced from the catalytic center and the interaction can be performed as required.

Different steps or functions of attachment factors can be simulated. One key might be a synthetic RGD-peptide

(commercially produced) or similar recognition site of an attachment protein. Another approach could be to simulate GAGs (see above). As GAGs have different functions, it might be easier to achieve the desired effect.

For industrial processes, it is not necessary to know exactly how the artificial biomolecule works, as long as it does so. There have already been a few known substances discussed in the international scientific community. Different dextran derivatives are capable of increasing or decreasing the adherence of cells. Barabino *et al*. (22) found that anionic polysaccharides inhibit adhesion of sickle erythrocytes to the vascular endothelium and result in improved hemodynamic behavior. Dee *et al.* and Donaldson *et al*. (23, 24) used dextran sulfate as adherence-decreasing substance to induce single cell formation in insect cell culture. Zanghi *et al*. (25) tested dextran sulfate for the ability to reduce clumping of CHO cells in a suspension culture system. A more distinct work with engineered dextran derivatives has been presented by the group of J. Jozefonvicz (21) (Institut d'Oncologie Cellulaire et Moleculaire Humaine (IOCMH), Bobigny, France). They tested different artificial dextran derivatives for their ability to generate chemical functions, thereby mimicking natural ligands.

Carboxymethyl benzylamide sulfonate dextrans are especially studied for heparin-like properties (25, 27). However, these groups of engineered substances are also capable of performing other tasks *in vitro*, such as inhibiting tumor proliferation (28) or influencing the production of collagen by the extracellular matrix (29). If the production of collagen can be inhibited, it might be also possible to induce collagen expression (30) with the effect that cells produce their own "extracellular matrix" on microcarriers. If so, the collagen-inducing biomolecule could be added to the medium directly after inoculation of a microcarrier process until a steady state is achieved. At this point, the cells adapt their micro-environment as required and the medium composition can be switched to production composition, resulting in a very stable and robust bioprocess (31). However, if stimulation of attachment protein production is not achievable via artificial biomolecules, a flow cytometry sorting for cells that produces higher amounts of the desired proteins might be a solution (32). For simulating GAGs, it seems that all their functions can be mimicked by artificial polysaccharides.

A completely different approach to manipulating the adhesion of cells is to use ions or different surface charges and charge densities (33). As mentioned earlier, Ca^{2+} and Mg^{2+} are cofactors for many attachment proteins and also for GAGs (34–39). Therefore, the Ca^{2+} concentration in media is often reduced to decrease adherence of cells cultured in suspension mode, or increased to culture anchorage-dependent cells on microcarrier beads (40). However, in addition to these two, more or less every other cation can modify the normally negatively charged culture surface. This fact must be taken into consideration when searching for adherence-influencing substances.

2.1.1 Adhesion to microcarriers

The adhesion of cells to culture surfaces is fundamental to both traditional monolayer culture techniques and to microcarrier culture. Since the proliferation of anchorage-dependent cells can only occur after adhesion to a suitable culture surface (41), it is important to use surfaces and culture procedures that enhance all of the steps involved in adhesion.

Attachment can be divided into four different phases. The first phase comprises a slight attachment to the surface. The second phase shows flattened but still spheroidal cells, where the cell is significantly more adherent due to the increased contact area and bond density. Cells in the third phase are significantly less adhesive than cells in phase two because of the reduced number of bonds. Cells in the fourth phase are fully attached and extremely flat (Fig 7) (42). Adhesion of cells in culture is a multi-step process and involves a) adsorption of attachment factors to the culture surface, (b) contact between the cells and the surface, (c) attachment of the cells to the coated surface, and finally (d) spreading of the attached cells (41, Fig 7).

The entire process involves divalent cations and glycoproteins adsorbed to the culture surface. Under normal culture conditions, attachment proteins vitronectin and fibronectin originate from the serum supplement in the medium. MHS is synthesized by the cells. The culture surface must be hydrophilic and correctly charged before adhesion of cells can occur (41). All vertebrate cells possess unevenly distributed negative surface charges (43) and can be cultured on surfaces that are either negatively or positively charged (41, 44, 45, 33, 46). Examples of suitable culture surfaces bearing charges of different polarities are glass and plastic (negatively-charged) and polylysine coated surfaces or Cytodex 1 microcarriers (positively-charged). Since cells can adhere and grow on all of these surfaces, the basic factor governing adhesion and growth of cells is the density of the charges on the culture surface rather than the polarity of the charges (33, 47).

Two factors in a culture medium are essential for cells to adhere to culture surfaces: divalent cations and protein(s) in the medium or adsorbed to the culture surface (41). Many established and transformed cell types secrete only very small amounts of fibronectin and thus require a fibronectin or serum supplement in the culture medium before adhesion occurs (48, 49). Certain types of cells, such as diploid fibroblasts, can secrete significant quantities of fibronectin and therefore do not require an exogenous source for attachment (50, 51). When initiating a culture, it is usual practice to let the culture surface come into contact with medium containing serum before cells are added to the culture. Culture medium supplemented with 10% (v/v) fetal calf serum contains approximately 2–3 µg fibronectin/mL (52), and a large proportion of the fibronectin adsorbs to culture surfaces within a few minutes (48). Serum-free media often require addition of fibronectin (1–50 µg/mL) before many cells can attach to culture surfaces.

Culture procedures affect the rate at which cells attach to surfaces. In the case of microcarrier culture, microcarriers and cells are often in a stirred suspension. Under such conditions, attachment of cells to Cytodex usually occurs to the same extent as with static culture systems. However, with some cell types, an initial static culture period is required so that all steps of adhesion (Fig 7) are fully completed.
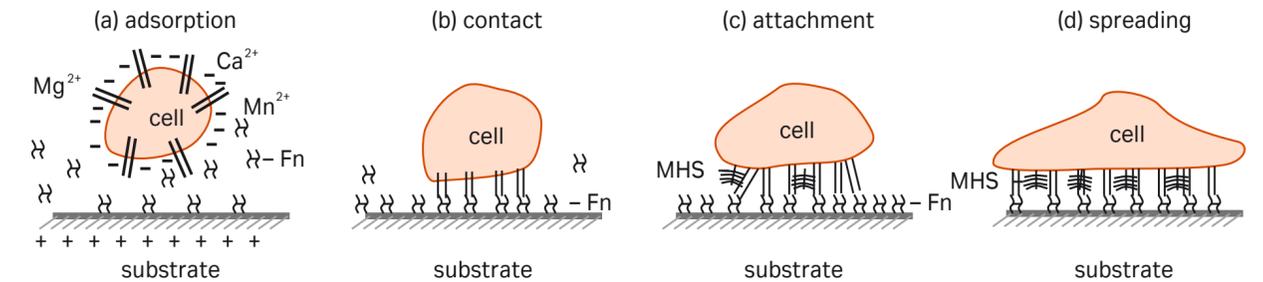
Fig 7A. Schematic drawing of the adhesion process in 4 phases (Fn- fibronectin, MHS- multivalent heparan sulfate).
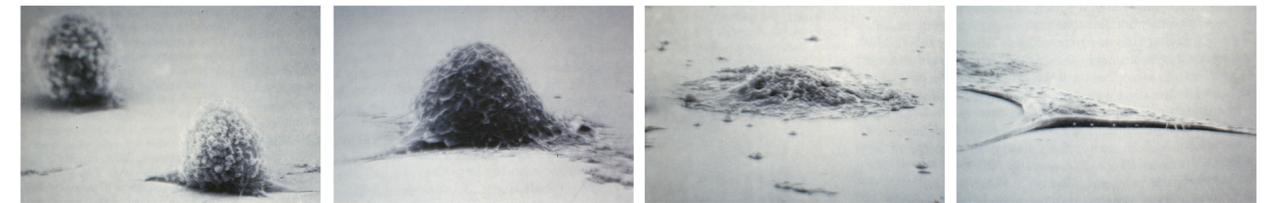
Fig 7B. Microscopic pictures of the 4 phases.

The design of microcarrier culture procedures for each type of cell is closely related to the adhesion properties of the cell and the rate at which all steps of adhesion are completed. Ways of determining optimal procedures for individual cell types are discussed in sections 8 and 10. Figure 8 illustrates the close attachment of cells to Cytodex.

For an adherent cell to quickly attach to a surface, it needs to be round (have a disrupted cytoskeleton) and have exchanged its cell surface receptors for newly produced ones. It is therefore necessary to have trypsinized cells in a single cell suspension (trypsin also acts as a growth factor). Work has shown that when the surfaces on which cells grow are degraded, the cells remain flat and retain their cell-cell contacts. These cells did not attach for several hours when seeded onto a cell culture surface. When they did attach, the distribution over the surface was very uneven due to clumping. This resulted in an inhomogeneous cell distribution on the carriers and a slower growth rate.

Normal and transformed cells seem to adhere in slightly different ways (Section 5.1.3). *In vitro*, adherent cells bind to extracellular matrix components; type I and IV collagen, fibronectin, vitronectin, laminin, chondronectin, thrombospondin, heparan and chondroitin sulfate.

The "normal" *in vitro* adhesion process is considered to occur via integrin receptors primarily binding to fibronectin (M_r 220 000) and vitronectin (M_r 65 000). The cells in turn bind to these proteins via the integrin receptors, with Ca^{2+} and Mg^{2+} ions as cofactors. A minimum of 15 ng/cm^2 of adsorbed fibronectin is required for BHK cell attachment. Fibronectin binds easily to gelatin, which is why gelatin gels are used for the affinity purification of fibronectin. It is also the reason why gelatin is a good cell culture substrate and used to manufacture microcarriers.

Vitronectin is considered to be more potent than fibronectin and active at even lower concentrations. Vitronectin is also called serum-spreading factor and has been shown necessary for the spreading-out phase of cells. These proteins are also produced by a number of cell lines. Other integrin receptors present on the cell surface are the collagen and laminin receptors. These proteins are not usually present in serum, but are sometimes added to coat the cell culture surface.

Cells can adhere to a wide variety of materials: glass, various plastics, metals (stainless steel 316, titanium used in implants), dextran, cellulose, polylysine, collagen, gelatin and numerous extracellular matrix proteins (see above).

Borosilicate glass is normally negatively charged. Attachment to it can be increased by treating the surface with NaOH or by washing with 1 mM Mg acetate.

Plastics used in cell culture include polystyrene, polyethylene, polycarbonate, Perspex™, PVC, Teflon™, cellophane, and cellulose acetate. These organic materials are made wettable by oxidation, strong acids, high voltage, UV light, or high-energy electron bombardment rendering them negatively charged. One major drawback of plastics is that they do not normally withstand autoclaving. Poly-d(l)-lysine is an artificial molecule that can also induce attachment when coated onto surfaces.

It appears that it is the charge density and not the type of charge that is important for attachment (33). Cells that grow attached on plastic surfaces should readily attach and grow on carriers.
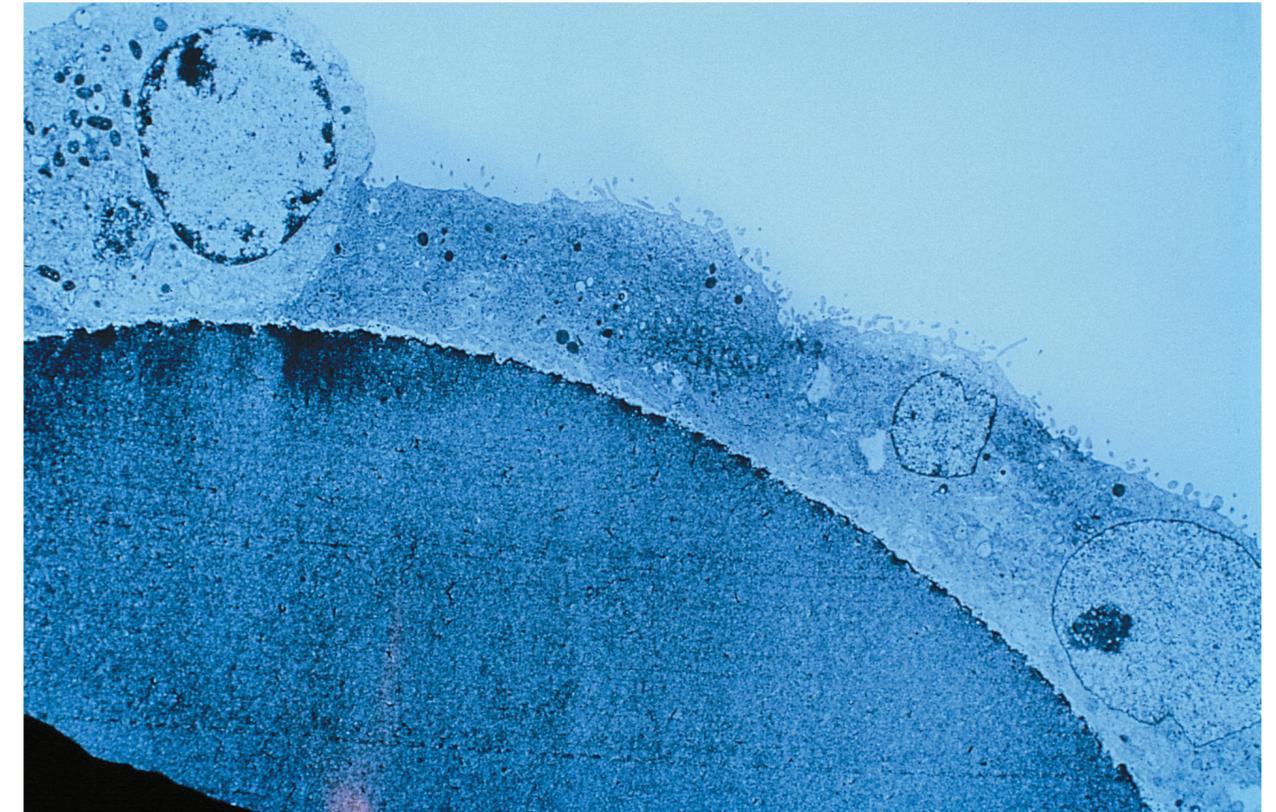
Fig 8. Transmission electron micrograph of pig kidney cells growing on a Cytodex microcarrier. (Original photograph by B. Meigneir and J. Tektoff, IFFA-Mérieux. Lyon, France, reproduced by kind permission.)

2.2 Immobilization principles

Immobilization was first described as early as 1923 by Carrell in the paper "Immobilisation of animal cells" (53). Today, biocatalysts (cells) are defined as immobilized when they are restricted in their motility by chemical or physical methods while their catalytic activity is conserved. Different immobilization techniques include binding biocatalysts to each other (aggregates) or on carriers, physical entrapment in a polymeric matrix or through membrane separation (Fig 5).

Immobilization allows heterogeneous catalysis, in contrast to catalysis in which substrate and biocatalyst (cells or enzymes) are homogeneously distributed. An economically viable separation of suspended biocatalysts from product is hardly possible. However, the immobilization technique does enable preliminary separation of product and biocatalyst, even during upstream processes. Immobilization also creates and maintains a high density of cells in a small volume (Table 3).

Table 3. Cell densities in different immobilization systems

Culture system	Cell density
Suspension	-10^6/mL
Cell retention	-10^7/mL
Cell immobilization	-10^8/mL
Ascites	-10^8/mL
Tissue	-2×10^9/mL

Another major advantage is that the medium feed rate is not dependent on the growth rate of the cells. The higher throughput of medium guarantees higher volumetric productivity. In fact, some cell lines cannot grow or produce the desired product without immobilization.

Butler (54) calculated the surface to volume ratios of different immobilization systems. Roller bottles had a ratio of 1.25, packed beds (with spherical carriers) 10, and artificial capillaries 30. Microcarriers (25 g/L) had 150, by far the best ratio.

2.3 Materials

Materials are important because of their chemical, physical, and geometrical effect on the carrier. For example, they influence toxicity, hydrophilicity, hydrophobicity, microporosity, mechanical stability, diffusion of oxygen or medium components, permeability, specific gravity, and shape (form, size, thickness, etc.).

A wide variety of materials have been utilized to produce microcarriers: Plastics, (polystyrene, polyethylene, polyester, polypropylene), glass, acrylamide, silica, silicone rubber, cellulose (Fig 9), dextran, collagen (gelatin), and glycosaminoglycans. These materials can be formed into different shapes. Spherical is the most common, but fibers, flat discs, woven discs, and cubes are also found.

Carriers are usually positively or negatively charged. However, non-charged carriers are also available. These are normally coated with collagen or gelatin, or have fibronectin or fibronectin peptides coupled to the surface. Glycosaminoglycan microcarriers are slightly negatively charged. Cells bind directly to the collagen, fibronectin or fibronectin peptides and to the glycosaminoglycan microcarriers (Section 2.1). Unfortunately, protein-coated and glycosaminoglycan carriers cannot be autoclaved as the protein structures are destroyed. However, gelatin (denatured collagen) can be autoclaved. Gelatin also has a very high affinity for fibronectin, which is why it is so suitable as a cell culture substrate.
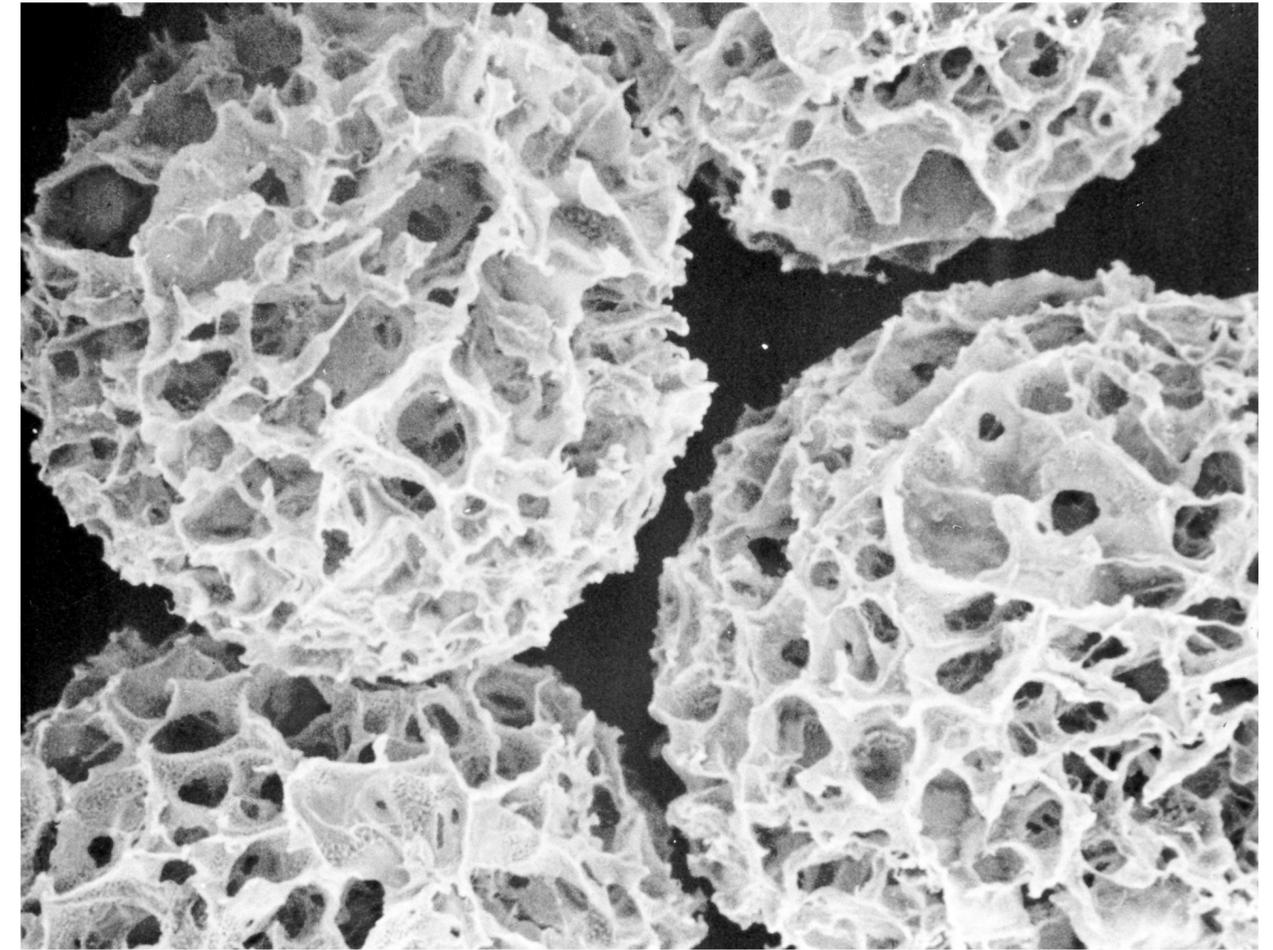
Fig 9. SEM picture of empty Cytopore macroporous microcarrier.

2.4 Size, shape and diffusion limits

The diameter of the different carriers varies from 10 µm up to 5 mm. The smaller are best suited for stirred tanks, whereas the higher sedimentation rates of the larger make them suitable for fluidized and packed beds. The smaller the carriers, the larger the surface in the settled bed volume because of the smaller void volume between them. The ideal size for smooth microcarriers is 100–300 µm. A very narrow size distribution is most important for good mixing in the reactor and an equal sedimentation of the beads during scale-up steps in large-scale processes. Emulsion and droplet techniques give round carriers.

Macroporous carriers are on average bigger because their pores may be up to 400 µm wide. A large pore size has to be balanced against the disadvantages of bigger particles, such as diffusion limits and higher shear stress on the outer surface.

Mass transfer in the immobilized cell aggregate is a significant problem in immobilized cultures. The poor solubility of oxygen in the medium at 37°C and the high consumption rate of the cells make it a marker for limitations in the cell aggregate. Keller (55) reports that in high-density cell cultures (up to $2 \times 10^{14}/m^3$ in the cell layer), an oxygen consumption rate of 5×10^{-17} mol/cell and second, and a medium volume that is 10-fold the cell mass, the oxygen is consumed within 3 minutes.

The single oxygen molecule has to overcome three barriers before it reaches the cell in the middle of the carrier: firstly transport from the gas phase into the medium, then transfer from the medium to the cell mass, and finally diffusion through the cell layers and consumption.

OTR (oxygen transfer rate) can be increased by increasing the volume-specific surface or using pure oxygen instead of air. The cells themselves should not be exposed directly to oxygen because of its toxic effect. However, the protection offered by macroporous structures allows the use of pure oxygen.

Many different methods of oxygenation are described. Bubble-free aeration (56), gassing with large bubbles (57) or via microbubbles (58, 59) are all used in high-density immobilized cultures.

Depending on the size of the carrier, the oxygen concentration on its outside must be increased. Carrier particles up to 500 µm in diameter can be supported with an oxygen tension of about 10% (Fig 10). If the particles are larger than 900 µm, limitations occur if the oxygen tension is below 35% (55). Griffiths (60) found sufficient oxygen penetration into cell layers up to 500 µm thick.

(A)

(B)
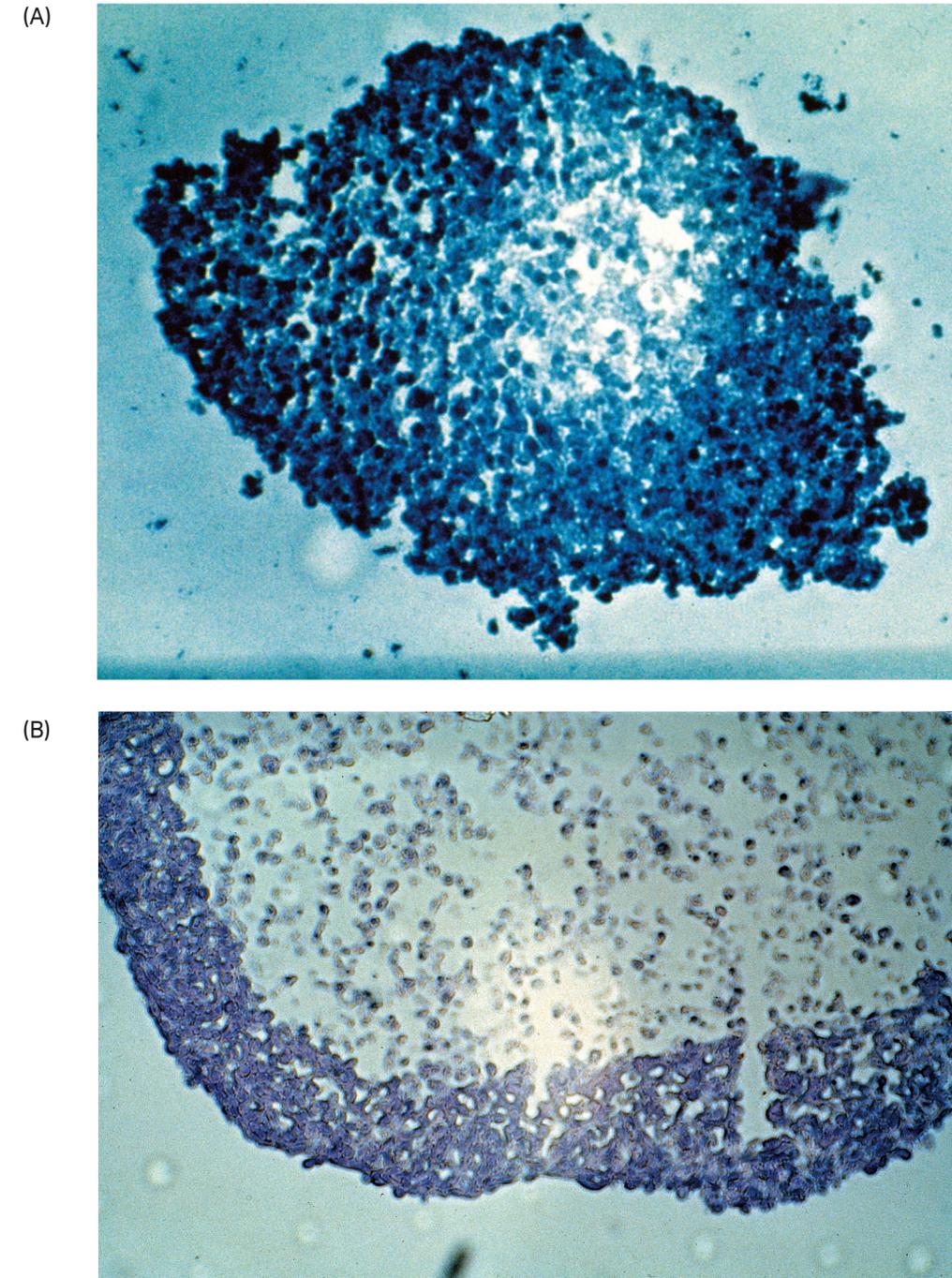
Fig 10. Viability stain of a small aggregate of 500 µm size (A) and 4000 µm size (B). The cells from the small aggregate are viable in the center, whereas the center of the large aggregate is nectrotic (lack of nutrient diffusion).

2.5 Specific density and sedimentation velocity

Smooth microcarriers in stirred tank reactors have a specific density just above the medium between 1.02 and 1.04 g/cm^3. Materials or material mixtures used to produce macroporous microcarriers have specific densities between 1.04 and 2.5 g/cm^3. However, sedimentation velocity is a better parameter to show the suitability of a microcarrier in a specific reactor type. This is because not only specific density but also size and shape influence the sedimentation velocity. Velocities lower than 30 cm/min do not create enough circulation and mixing for efficient nutrient supply throughout the carrier bed in fluidized bed technology (55). Adherent cells also tend to form bridges between microcarriers in fluidized beds. Higher sedimentation rates (150–250 cm/min) will prevent such bridges from forming. Sedimentation velocities are summarized in Table 4.

Table 4. Table of microcarrier sedimentation velocities

Microcarrier	Sedimentation velocity cm/min	Specific density
Cytodex 1	12–16	1.03
Cytodex 3	12–16	1.04
Cytopore 1	13–19	1.03
Cytopore 2	13–19	1.03
Cell	0.04	1

2.6 Rigidity and shear force

Microcarrier rigidity is important in long-term cultures. The materials used should withstand the organic acids and proteases found in culture supernatants. Abrasive carriers made of brittle materials such as glass or ceramics could harm cells, valves and bearings, and cause problems when filtering culture supernatants.

In turbulent fluids, particle/particle collisions and particle/stirrer collisions are very energetic (61). In contrast to stirred tanks, the shear forces in fluidized beds are homogeneously distributed and impeller/carrier collisions are not possible. Shear forces in fluidized beds correlate with particle sedimentation velocity and reactor type. Keller (55) measured shear tensions of about 0.3–0.5 N/cm^2, which are far below the damaging shear tension for kidney cells (10 N/cm^2 (62)). Spier (63) transferred shear forces into wind velocities to demonstrate the force affecting the cells. The linear velocity should not be more than 0.3 km/h during attachment of anchorage-dependent cells, while a velocity of 95 km/h is necessary for cell detachment.

2.7 Porosity

Carriers can either be solid or microporous. Microporous carriers allow the cells to take up and to secrete material on the basolateral side of the cell. Molecules up to M_r 100 000 can penetrate these carriers (Fig 11). Note that when these microcarriers are entirely confluent, there can be a different environment inside the beads than on the outside!

The latest development in microcarrier technology is macroporous carriers (Fig 12) that allow cells to enter. Their average pore size is between 30 and 400 μm. As the mean cell diameter of single cells in suspension is about 10 μm, this allows cells easy access into the carriers. Macroporous carriers are also suitable for immobilizing non-adherent cell types. In this case, the cells are forced into the matrix and entrapped. Macroporous carriers give higher cell densities and are therefore normally used in perfusion culture.

The porosity of macroporous carriers is defined as the percentage volume of pores compared with the total carrier volume. It is normally between 60 and 99%.

In spite of the large number of microcarrier designs and types, very few are still commercially available. Even fewer fulfill industrial standards for large-scale manufacturing processes.

2.8 Cell observation

Microcarrier transparency is important for simple cell observation in a light microscope (Fig 29, plates 2, 5, 6, 9). In vaccine production especially, it is important to see the morphology of cells directly on the carrier to find the right moment to infect the cells or harvest the virus. Unfortunately, due to the size, three-dimensional structure and material of a number of microcarriers, the cells cannot be observed clearly with a light microscope. Furthermore, the scanning electron microscope is not suitable because of the long preparation time and the effect of dehydration on cell shape and morphology (Fig 29, plate 8).

However, confocal laser scanning microscopy (64) is an excellent tool for making cells visible in the pores of macroporous beads (Fig 13B). With this technique, it is possible to make optical sections through the carrier and create a three-dimensional reconstruction.

A viability stain with two fluorescent dyes (FDA fluorescein diacetate for living cells and ethidium bromide for dead cells) allows the viability of cells in three-dimensional structures to be estimated (65, 66) (Fig 13A). Other staining methods such as MTT (67) also make cells visible in macroporous structures.
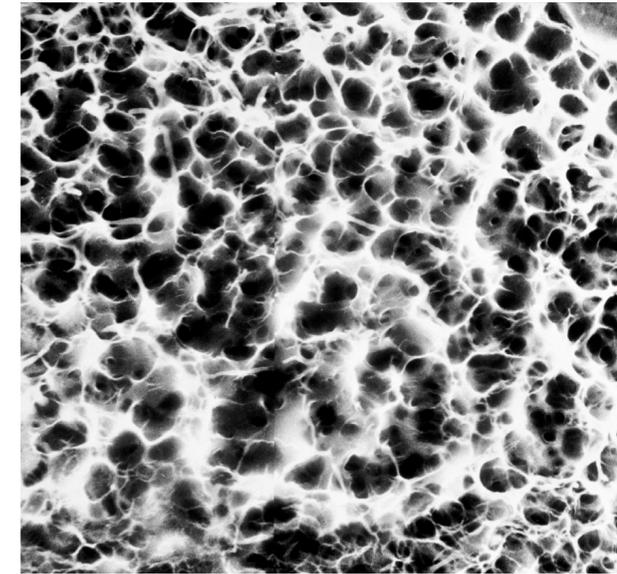
Fig 11. Microporous structure of Cytodex (pores are not available for cells).
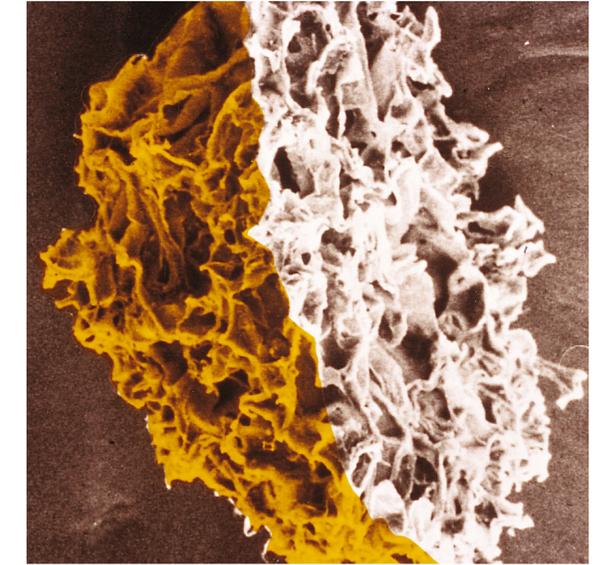
Fig 12. Cross-section through the macroporous Cytopore bead. The bead is porous throughout (95% porous).
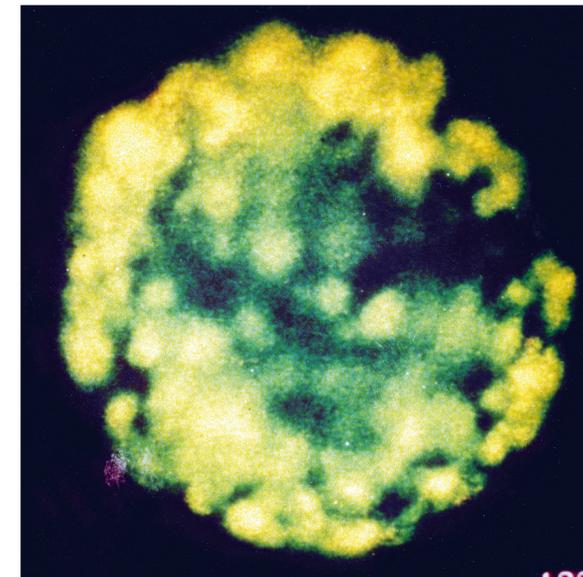
Fig 13A. Confocal laser scanning microscope photograph of FDA (Fluorescein diacetate) stained CHO cells covering Cytopore beads.
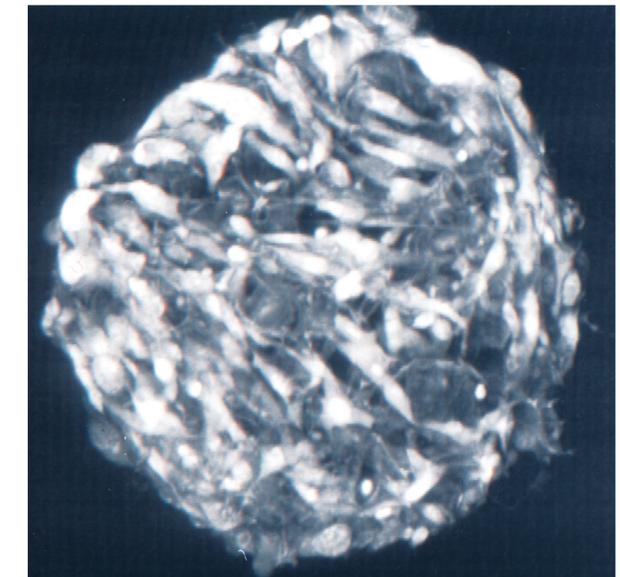
Fig 13B. Confocal laser scanning micrograph of acridine orange stained Vero cells on Cytopore beads.

03

Microcarrier technology

3.1 The development of microcarriers for animal cell culture

The idea of culturing anchorage-dependent animal cells on small spheres (microcarriers) kept in suspension by stirring was first conceived by van Wezel (68). In the first experiments, van Wezel (68) used the beaded ion exchange medium DEAE Sephadex™ A-50 as a microcarrier. This proved useful in initial experiments since it provided a charged culture surface with a large surface area/volume ratio, a beaded form, good optical properties and a suitable density. Glass spheres were not suitable because their high density required stirring speeds that were not compatible with cell growth (47, 68).

Several workers have suggested that the ideal microcarrier should have properties similar to those of DEAE Sephadex A-50 (46, 69, 70, 71, 72, 73). Other ion exchange media all proved to be inferior to this medium (44, 45, 46). Using DEAE Sephadex A-50 at a concentration of 1 mg/mL, van Wezel demonstrated that a homogeneous microcarrier system could be used for the large-scale culture of anchorage-dependent cells, including diploid human fibroblasts (68). This early work illustrated the potential of the microcarrier technique for producing virus, and later experiments established that this technique could be scaled-up for a variety of production processes (1, 71). Since the yield of anchorage-dependent cells depends on the surface area available for growth, it was believed that the maximum cell density (yield) in microcarrier cultures would depend on the microcarrier surface area (1). However, when the quantity of DEAE Sephadex A-50 exceeded 1–2 mg/mL, toxicity was encountered and there was not a proportional increase in cell yield (1, 70). This toxicity was manifested by the failure of many types of cells to survive the early stages of culture, long lag periods, and limited cell yields at the plateau stage of culture.

Explanations for this phenomenon have varied, but it is now known that the degree of substitution of DEAE Sephadex A-50 was not optimal for cell growth (44, 45, 69, 74). The toxicity was probably due to excessive ion exchange capacity in the micro-environment of the cell rather than too large a total exchange capacity in the culture (69). Although early culture experiments were not controlled or optimized for parameters such as plating efficiency, inoculation density, serum and culture medium, the work of Kuchler *et al*. (75), Inooka (76) and Horng and McLimans (44, 45) suggested that alterations in the ion exchange capacity of DEAE Sephadex A-50 could lead to improvements in cell attachment and growth.

The ion exchange capacity of DEAE Sephadex A-50 could be altered by changing the culture environment (e.g., ionic strength, pH), but such changes were very limited since cells require physiological conditions for growth. This problem was overcome by the development of microcarriers with a much lower degree of substitution than DEAE Sephadex A-50, and which also fulfilled the requirements for an optimal microcarrier (46, 69, 71, 73, 74). Table 5 shows the effect on cell growth of different degrees of substitution of Sephadex with DEAE-chloride.

Table 5. Effects of substitution on cell growth

Sephadex media	meq/g	1 g/L	2.5 g/L
DEAE Sephadex A-50	3.5	2	1
DEAE Sephadex A-50 + 0.01% CMC	3.5	3	2
DEAE Sephadex A-50 + celloidin	3.5	4	3
Sephadex G-50 + DEAE subst	1.8	4	4
Sephadex G-50 + DEAE subst	0.9	1	1

1 = bad, 2 = moderate, 3 = good, 4 = excellent

Using Sephadex as starting material and substituting the matrix with DEAE groups to 1.5 meq/g dry product, it was possible to achieve a microcarrier, Cytodex 1, that was suitable for the growth of a wide variety of cells (47, 74, 77, 78). Cytodex 1 avoided the toxic effects associated with DEAE Sephadex A-50, and concentrations well in excess of 1 mg/mL could be used with concomitant increases in cell yield.

This reduced-charge product was the first to allow the full potential of microcarrier culture to be exploited at culture volumes up to several hundred liters (10, 79, 80). It was specifically designed for animal cell culture and satisfied the general requirements for an optimal microcarrier (46, 47). Under correct culture conditions, the growth rate of most cells on Cytodex 1 is comparable to that achieved on plastic or glass culture surfaces (Fig 14).

The development of Cytodex 1 has taken into account the requirements for cell attachment (Section 2.1) and the procedures necessary for maximizing growth of a wide variety of cells in microcarrier culture (Section 8). Opportunities for microcarrier culture of animal cells have been increased further by the development of Cytodex 3 (Section 5.1.1 and 5.1.2). Since charged groups are necessary only for cell attachment, they need only be confined to the surface of the microcarriers. Cytodex 3 represents a new concept in microcarrier culture. Instead of using synthetic charged groups to promote cell attachment, it has a surface layer of denatured collagen. The surface upon which cells attach is thus similar to that found *in vivo*. Such a surface is important for maximum plating efficiency, growth and function of certain cell types and lends itself to unique possibilities for harvesting cells from microcarrier cultures (Section 8.6.1). Nilsson and Mosbach (81) have also examined this approach.

The relative properties and uses of the different types of Cytodex microcarriers are outlined in Section 5.1.

The next major step forward was macroporous gelatin microcarriers developed by Kjell Nilsson (82), which allowed growth inside the beads, thereby increasing cell density and protecting the cells. Young and Dean (83) then described the use of microcarriers for animal cells in fluidized beds. This development allowed the immobilization of both anchorage and suspension cells in high cell density production systems.

Cytopore was yet a further development that kept most of the properties similar to Cytodex but increased the surface area through a macroporous structure. Cytoline was developed for the fluidized bed application, which is why the particles were weighted with silica (84).
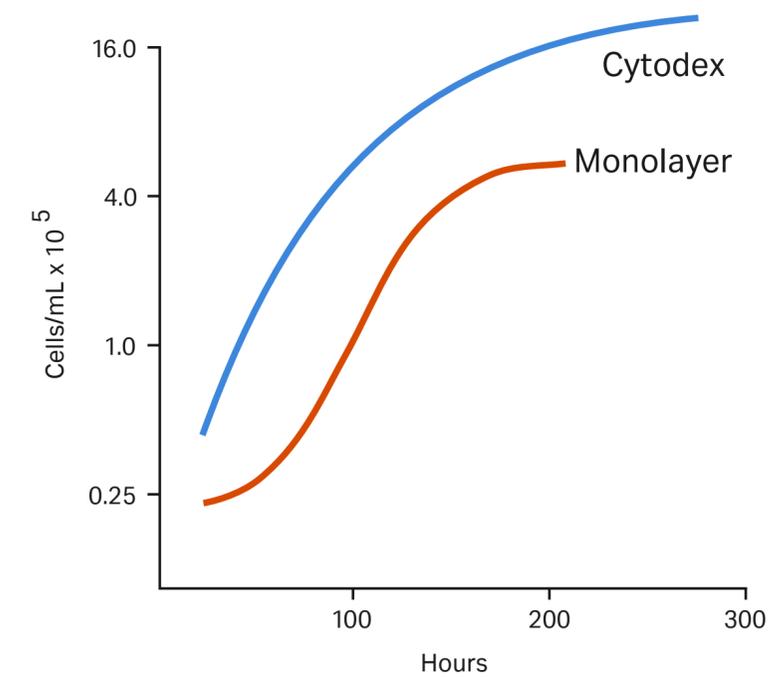
Fig 14. The growth of primary monkey kidney cells on Cytodex 1 microcarriers and in glass bottles. (Data from van Wezel, A. L., reproduced by kind permission.)

3.2 Advantages of microcarriers

Microcarriers have many advantages. They are essential when surfaces are needed for anchorage-dependent cells. They are also inexpensive (price/m^2). Microcarrier technology results in a homogeneous culture system that is truly scalable. Because of their large surface area to volume ratio, they occupy less space in storage, production and waste-handling. The surface also allows cells to secrete and deposit an extracellular matrix, which helps introduce certain growth factors to cells. Spherical microcarriers have short diffusion paths, which facilitates nutrient supply in general.

The extracellular matrix also gives cells support to build their cytoskeleton and to organize organelles intracellularly, both of which may increase the yield of functional product.

Microporous carriers (Fig 11) allow cells to create a micro-environment inside the beads. They also facilitate polarization and differentiation of cells. As the cells are immobilized on the microcarriers, it is easier to retain the cells in culture during perfusion. This separates cells from products. At high cell concentrations and perfusion rates, the residence time of the product at 37°C is very short. It can quickly be separated from the cells and cooled down.

Macroporous microcarriers offer additional advantages. They allow cells to grow in three dimensions at high densities which stabilizes the cell population and decreases the need for external growth factors. This makes it easier to use low serum, serum-free and even protein-free media, which, of course, cuts costs (Section 9.1.1). The high cell density confers more stability and improves the longevity of the culture, making macroporous microcarriers suitable for long-term culture. The structure also protects the cells from shear forces generated by the stirrer, spin filter and air/dO$_2$ sparging (85), which facilitates oxygen supply. Macroporous microcarriers can be used both for suspension (entrapment) and anchorage-dependent cells. Several culture technologies can be used, including stirred, fluidized and packed bed reactors.

The process advantage of macroporous carriers is that the high perfusion rates maintain a homogeneous environment. This not only ensures a sufficient nutrient supply but also removes toxic metabolites. Vournakis and Runstadler (86) note that a regular distribution of oxygen and other nutrients in the pores is secured through a "micropump". The convection stream through the fluidized bed creates a pressure drop over the carrier surface that causes medium to flow in and out of the carrier.

Changing media from fetal calf serum-containing to protein-free is a very time-consuming process or sometimes not possible. The immobilization technology using macroporous microcarriers helps reduce the adaptation time or even enables the use of protein-free media. The micro-environment created by the cells inside the pores (autocrine growth hormones) allow the cells to survive and grow without serum supplement (87).

3.3 Possible drawbacks

Some carriers have to be washed and prepared for use. Scale-up using cells harvested from microcarriers is more complex than suspension expansion. It is even more difficult to harvest cells from macroporous carriers. Due to the higher cell density, cell enumeration and harvesting are also more difficult. In addition, the high density makes it harder to effectively infect all cells simultaneously, especially using non-lytic viruses. Finally, large carriers with small pores may restrict the diffusion of nutrients to some cells.

3.4 Scale-up considerations

Scale-up starts with the creation or choice of cell line, the characteristics of which greatly influence the scale-up possibilities and technology choices! When choosing the cell line, bear in mind the production technology available and the final scale of operation. This is especially important if hardware investments have already been made. For example, scale and technology affect the stability of expression as well as the adhesive properties required by the cell. The higher the degree of transformation, the more difficult it is to get the cells to attach and spread onto surfaces. It may thus prove difficult to find a suitable "industrial" microcarrier if technology screening is left too late after designing the cell line.

When evaluating different microcarriers for production, consider the consequences of the choice at the final process scale. Normally, it is easier to handle a microcarrier that is autoclavable. It should also be possible to handle the material in the open, e.g., to subdivide lots more easily.

If mistakes are made during process startup, it helps if the microcarrier can be resterilized without loss of material. Note that it is more difficult to sterilize carriers at larger scale; an autoclave is no longer viable due to the large volumes. Normally, the microcarriers are then sterilized inside the bioreactor.

The type of microcarrier is intimately linked to the design of the bioreactor. Large, high-density microcarriers always have to be prepared inside the bioreactor. The size of the carriers will influence their transfer through valves and tubing during sterilization and harvesting. Size and density will also influence how easy it is to keep carriers in suspension (i.e., impeller and reactor design, stirrer speed, etc.) and how quickly they sediment to allow large volume media changes (fed-batch). These characteristics also affect how easy it is to keep back the microcarriers in a perfusion system (settling zone, spin filters).

Also consider if the microcarrier generates truly homogeneous cultures or causes heterogeneity. Homogeneous cultures are normally easier to scale up as they facilitate oxygen and nutrient supply, and make it easier to remove waste products.

The choice of solid versus porous microcarriers will impact on how easy it is to wash the cells during media changes from serum to serum-free media, and to wash and harvest cells from the carriers. Solid carriers are normally easier to handle in these respects. Macroporous carriers are the most difficult at harvest. Easiest to harvest are gelatin or collagen microcarriers, as it is possible to enzymatically degrade them and recover the cells.

The simplicity of a low-density system should be weighed against the demands and complexity of high-productivity, high cell density cultures. Another aspect to consider is inactivation of the carriers after production. This is especially important when producing hazardous agents. Once again, it is advantageous if the carriers can be autoclaved prior to disposal.

Positively charged carriers will stick to non-siliconized glass and stainless steel. This can be overcome during cleaning by washing with high-pressure water, automatically via spray balls or manually using high-pressure washes. This "stickiness" can be decreased by changing pH to decrease the charge and increasing the ionic strength by adding salt to the washing solution.

The material used to produce the carriers will influence waste disposal. Sometimes the material is naturally degraded in nature.

One further aspect to consider is whether the microcarrier is made of a material that is used in downstream chromatography processing. Process validation will be facilitated if it is.

Finally, find out whether the microcarrier is an established product that is already registered with regulatory authorities for similar production processes. If so, it should help when registering new processes.

3.5 Choice of supplier

Important aspects to consider when evaluating suppliers of microcarriers include their production capacity, i.e., the available batch size, which affects purchasing as well as testing during full-scale operation. Also evaluate what function and quality testing the supplier carries out, and if a certificate of analysis is available for each batch. This helps minimize batch-to-batch variation and ensure process consistency. Normally, at least three different lots should be evaluated before scale-up. The availability of product stability and leakage studies is also important.

Regulatory issues to consider include whether the supplier works according to ISO 9000 and/or cGMP, and if it is possible to audit the company. Ask if Regulatory Support Files or Drug Master Files are available for the microcarrier (Table 6). Support in the form of application work, product information, troubleshooting and validation support is always useful. Don't neglect to check the financial stability of the manufacturer as well; this is important in helping secure supply for the lifetime of the process. Finally, the price of the microcarriers is naturally a key issue. Remember, however, that the main part of Costs Of Goods Sold is fixed costs!

Table 6. Comparison of Regulatory Support and Drug Master Files

Regulatory Support File	Drug Master File
+ Information directly to customer	- Information directly to FDA
+ Updated automatically when new information available, information accessible to customer	- Updated annually, information not accessible to customer
+ World-wide support	+ Well accepted system
- No recognized approval	- No recognized approval
- No manufacturing information	+ Contains manufacturing information

04
Applications

Well over 600 publications reflect the many successful applications of microcarrier technology and the great number of different cell lines cultured. Today, its main industrial use is to produce vaccines, vectors for gene therapy, natural and recombinant proteins and, increasingly, monoclonal antibodies. An interesting minor application is its use in artificial organs (livers).

The number of applications run using microcarrier technology may be influenced by the choice of producer cell and its glycosylation pattern. If more natural adherent cell lines are chosen, applications of the technology will increase greatly.

Today's applications fall into three categories: a) high-yield production of cells, viruses or cell products, b) *in vitro* cell studies, and c) routine cell culture techniques.

4.1 Vaccines

A vast majority of vaccine producers in Europe, and many others world-wide, use surface microcarriers to produce live attenuated or inactivated vaccines for human and veterinary use (Fig 15, Fig 16) (10, 88). Recently, they have started to produce viral vectors used in gene therapy, adenovirus and murine retroviruses; both lytic and non-lytic viruses are produced. As the cells are eventually killed, natural-batch or fed-batch processes are used. Normally, low cell densities are cultured in stirred tank cultures for this purpose. One novel application is to use diploid MDCK epithelial cells to produce influenza vaccine (Section 9.1.3) (89).
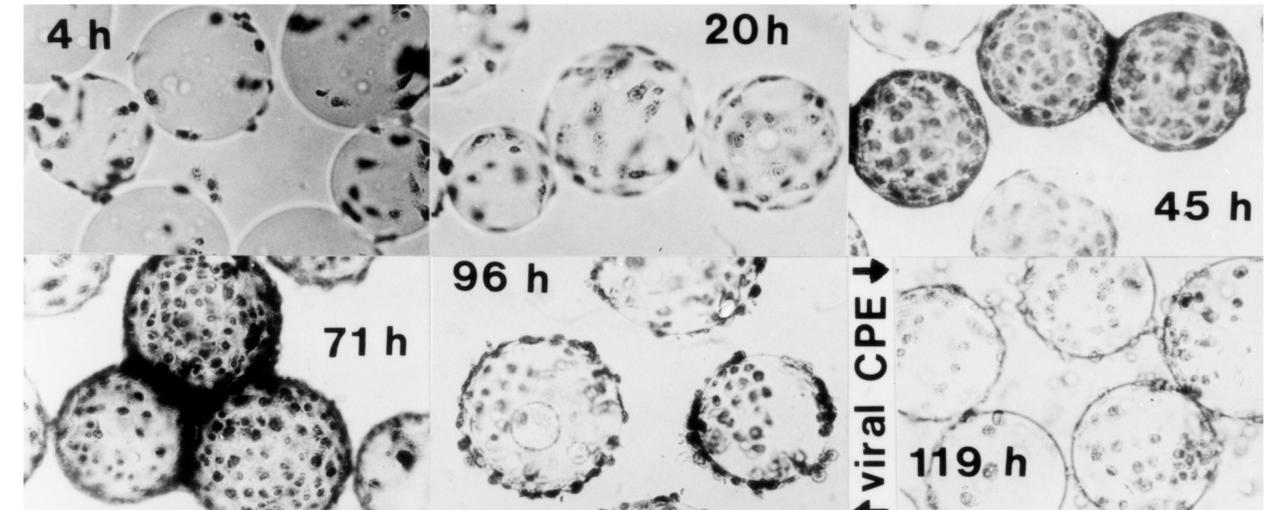
Fig 15. Culture of Vero cells on Cytodex microcarriers used for the production of *Herpes simplex virus* (HSV-2). The culture was infected with HSV-2 after approx. 50 h. CPE-cytopathic effect. (Original photograph by B. Griffiths, CAMR, Porton Down, UK, reproduced by kind permission.)
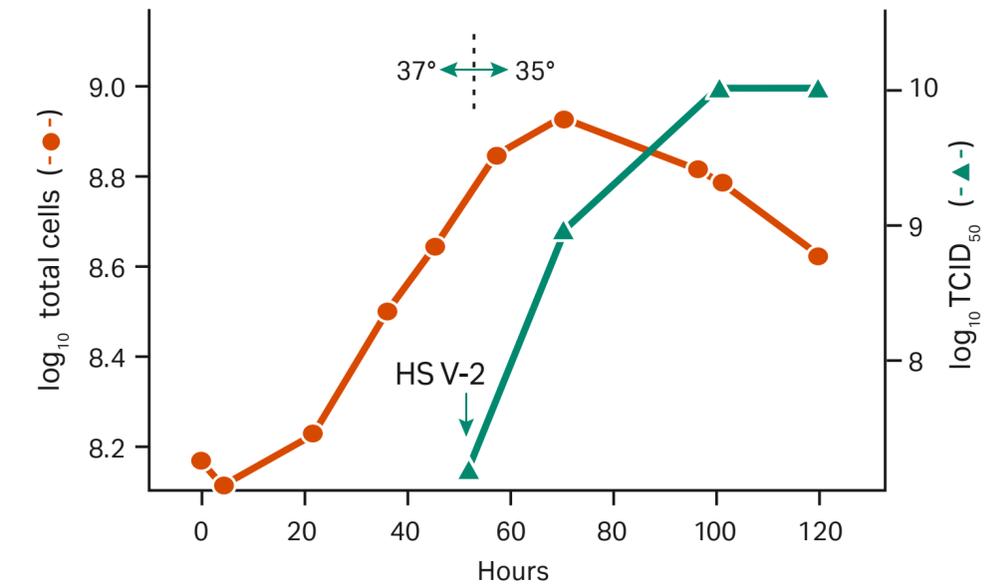
Fig 16. The growth of Vero cells and *Herpes simplex* virus (HSV-2) in stirred cultures containing Cytodex microcarriers. Figure 15 shows photomicrographs of this culture. (Griffiths, B., Thornton, B., McEntee, I., *Eur. J. Cell Biol.* **2,** 606 (1980) reproduced by kind permission.)

A microcarrier workshop (2002 in Rome) confirmed the many applications in the vaccine industry (Table 7).

Table 7. Summary of applications in vaccine industry

Company	Article	Journal/Patent	Microcarrier
Baxter, Austria	Influenza vaccine, smallpox	Microcarrier workshop 2002	Cytodex 3
Baxter, Austria	Transfected Vero cells, protein-free, HAV-vaccine, 100 L perfusion mode. 100 liter fermenter running for 200 days	Microcarrier workshop 2002	Cytopore 2
NVI, Netherlands	IPV production	Microcarrier workshop 2002	Cytodex 3
Xenova	Disc Herpes simplex virus 2	Microcarrier workshop 2002	Cytodex 3
Institute Pasteur Tunis	Rabies vaccine in BHK and Vero cells	Microcarrier workshop 2002	Cytodex 1
Aventis	JEV, Vero cells up to 2000 L cultures	Patent	Cytodex 1
Connaught-Lab	Comparison of large-scale serial subcultivation of WI-38 versus MRC-5 on Microcarriers WI-38 and MRC-5 in 50 L to 1000 L culture	In-Vitro (1993)	
Connaught-Lab	Large-scale serial subcultivation of MRC-5 cells on microcarrier	In-Vitro (1992)	
GSK	High-density microcarrier culture for viral vaccine production, Vero cells, polio virus	Adv. Anim. Cell-Biol. Technol. Bioprocesses (1989)	Cytodex 3
Solvay Pharmaceuticals, Netherlands	InfluvacTC: A safe Madin Darby Canine Kidney (MDCK) cell culture based influenza vaccine	Williamsburg Bio Processing Foundation, Nov 16–19, 1998	Cytodex 3

4.1.1 Production of viruses and vectors

Cells cultured on microcarriers are often used as substrates for the production of viruses or cell products, and the method is compatible with standard production procedures; Cytodex can be used for the production of all substances that can be produced in animal cell culture. A wide variety of viruses can be produced using Cytodex, including viruses sensitive to growth in suspension cultures, e.g., Herpes. The microcarrier system allows cultivation of large quantities of virus in compact culture units and provides an improved system for the production of many vaccines (1, 79, 90, 91). Vaccines produced in the microcarrier system include polio, rubella, rabies, influenza, Japanese encephalitis, RSV and foot-and-mouth disease (FMD) vaccines (1, 10, 79, 80, 90, 91, 92, 93). Figure 16 and Figure 17 illustrate the growth of Vero cells and the production of Herpes simplex virus on microcarriers. Viruses that have been grown in cultures using Cytodex microcarriers include, among others:

Adenovirus	Marek's	Respiratory syncytial virus
Bovine rhinotrachteitis	Measles	Rous sarcoma
Endogenous C-type	Papova virus	Rubella
Equine rhinopneumonitis	Parvovirus	Sendai
Foot-and-mouth	Polio	Simian virus 40
Group B arboviruses	Polyoma	Sindbis
Herpes	Pseudorabies	Vaccinia
Influenza	Rabies	Vesicular stomatitis

The advantages of microcarrier culture for vaccine production include increased productivity, lower costs and reduced contamination when compared with other cell culture methods. Sinskey *et al.* (94) observed that the volumetric productivity of Sindbis virus in microcarrier culture is in excess of 50-fold greater than that of roller bottles. Van Wezel *et al.* (91, 92) have developed a "Unit Process" for producing polio and rabies vaccines using Cytodex, and the productivity and efficiency of such a system is illustrated in Table 8. An example of a cell culture scheme for the production of inactivated polio vaccine is shown in Figure 17. Serial cultivation on Cytodex reduced the requirement for a source of primary cells and provided a production culture of 650 liters.

Table 8. Processing of polio virus type I from microcarrier cultures using Cytodex

Step	Vol L	D-antigen DU/mL	Recovery %	Albumin µg/mL	IgG µg/mL
Virus suspension	240	76	100	n.d.	n.d.
Clarification	248	64.2	87	1.000	300
Concentration	1	17.530	96	> 30.000	> 30.000
Gel filtration	4.5	3.465	85	0.23	2.0
Ion-exchange	4.5	3.465	85	0.03	< 0.23
Sterile filtration	7.5	1.964	81	< 0.03	< 0.23
Monovalent vaccine	7.4	1.753	71	< 0.03	< 0.23

PN content: after gel filtration, 40 mg/mL; after ion exchange, 8 µg/mL. Gel filtration was performed with Sepharose™ 6B in a Pharmacia K 215/100 column, and ion-exchange chromatography was performed with DEAE Sephadex A-50. (van Wezel, A.L., van Herwaarden, J.A.M., van de Heuvel-de Rijk, E.W., Devel. Biol. Stand. **42**, 65, (1979) by kind permission of the authors and the publisher.)

Von Seefried and Chun (90) reported high yields of polio virus having high infectivity (8.84 $\log_{10}$ TCID$_{50}$/mL or more) when using human fibroblasts (MRC-5) growing on Cytodex. Vero cells growing on Cytodex have been used for the production of a stable polio vaccine from culture volumes of 140 liters (80). Polio virus production can also be taken as an example to illustrate yields of virus from microcarrier cultures, as summarized in Table 3. Giard *et al.* (2) reported that the yield of polio type III virus from microcarrier cultures (6.5 pfu/cell) was greater than the yield from roller bottles (4.0 pfu/cell). Similarly, Mered *et al.* (95) observed that the yield of polio virus/cell was greater from microcarrier cultures than from culture flasks.

Cytodex has been used to produce rabies vaccine by multiple harvests from primary dog kidney cell cultures (91, 93). The infective titer of the harvests was 6.0 ± 1.0 $\log_{10}$ LD$_{50}$/mL in mice. Twenty-one FMD vaccines of good quality and with long storage life were produced from pig kidney cells growing on Cytodex (10). The vaccines gave good protection of animals with no abnormal local reactions, and it was not necessary to concentrate the antigen (10).
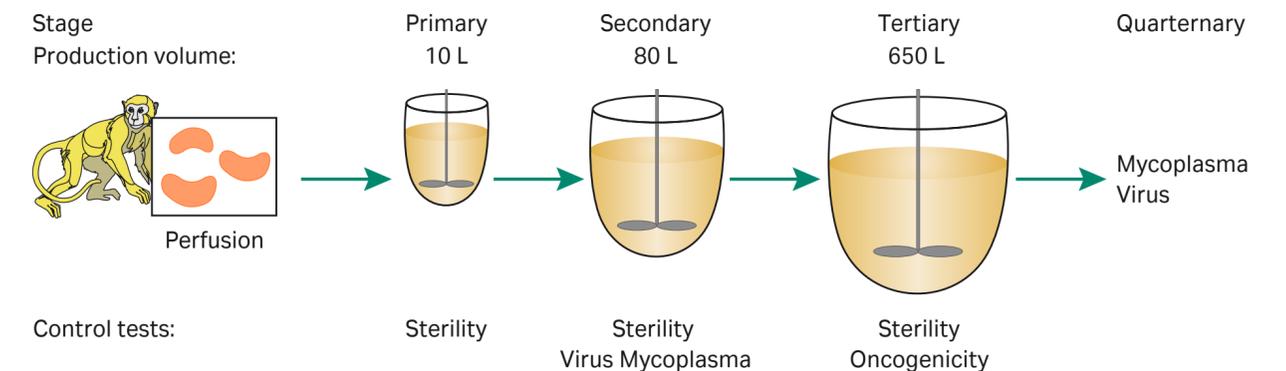
Fig 17. Scaling up microcarrier cultures for the production of large numbers of cells. Example illustrates the subcultivation system for production of polio vaccine from monkey (Cynomolgus) kidney cells growing on Cytodex microcarriers. (Adapted from van Wezel, A.L., van der Velden-de Groot, C.A.M., van Herwaarden, J.A.M., *Develop. Biol. Standard* **46**, 151 (1981).)

Spier and Whiteside (96) have compared the production of FMD virus from BHK cells grown on microcarriers and in suspension. Microcarrier culture of FMD virus Type 0 gave a virus suspension with higher infectivity and complement-fixing activity than suspension culture. The complement-fixing activity of FMD virus Type A from microcarrier cultures was at least five times that obtained from suspension cultures (96). A large-scale controlled fermenter and Cytodex have been used for the prolonged culture of cells persistently infected with papova virus (97). Manousos *et al*. (98) studied the production of oncornavirus in long-term microcarrier cultures and noted that one advantage of this technique was that addition of new microcarriers to confluent cultures caused a new wave of cell growth and virus production. The production of other types of viruses in microcarrier culture is described in references 2, 94, 99.

Microcarrier culture provides a potential method for the mass-production of fish virus vaccines. Nicholson observed that the production of infectious pancreatic necrosis virus from microcarrier cultures (44.5 $TCID_{50}$/cell) was nearly three-fold greater than production of virus from culture flasks (16.0 $TCID_{50}$/cell).

Dr. M. Tanner (Werthenstein Chemie AG, Switzerland) presented a large-scale production process of rAdV on 293 cells grown on Cytodex 3 carriers at the first microcarrier workshop, Rome 2002. Scale-up was performed starting from one T-75 culture, via one T-500, three T-500, one CF-10 (cell factory), four CF-10, four CF-40, twelve CF-40, and a 500 liter bioreactor to the 2000 liter production scale reactor. The optimal scale-up protocol would be replacement of the four CF-40 and twelve CF-40 steps by one 20 liter and one 100 liter bioreactor step. To keep a high standard of hygiene, large-scale peristaltic pumps were used for all transfers. The workers used 2–4 g carriers per liter and inoculated 8–20 cells/carrier. Optimal conditions were pH 7.2–7.4 and pO_2 about 40%. They worked in a perfusion mode to maintain a relatively low lactate concentration. The carriers were retained using a spinfilter, and the maximal perfusion rate was one working volume per 2 h.

As a critical scale-up parameter, a constant power input was used. The agitation speed in the 2000 liter reactor was 15–25. This speed is normally increased by a factor of three for detaching the cells during trypsinization, which is relatively easy to perform. The medium containing 10% FCS was diluted to a FCS content of 1%, trypsin was added, and as soon as 90% of the cells had detached, serum was added to inactivate the trypsin activity. The cells were then transferred to the recipient reactor (next size), which already contained equilibrated new Cytodex 3 carriers.

4.2 Natural and recombinant proteins

A number of processes for naturally produced proteins are based on the culture of diploid cell lines on surface microcarriers in stirred tanks. Most new recombinant proteins are expressed in CHO cells. They do attach and grow initially on surface microcarriers, but after some days aggregate and start to fall off. The majority of CHO cells have been adapted for suspension culture and grown at fairly low cell densities in stirred tanks in batch or fed-batch cultures. Lately, however, some processes utilizing macroporous microcarriers to increase cell density have been developed. In some cases, this has led to increased productivity. In the paper of Shirokaze (295), a doubled productivity of r-Il4 could be obtained with immobilized culture compared to suspension. Production was measured by ELISA over an 11-day period. The total productivity in suspension in 10% calf serum was 2 mg; in serum-free, 1.8 mg. In immobilized culture, the figures were 3.8 and 3.2 mg respectively. The processes ran as perfusion cultures utilizing spin filters in stirred tanks. In addition, some processes are being set up using r-CHO cells in fluidized bed cultures with up to 100 liter reactor volumes (60 liter fluidized bed volume).

Interferon has been produced in high yield from microcarrier cultures. The first report (100) described yields of 4×10^3 IU HuIFNβ/10^6 human fibroblasts. A more detailed study examined various parameters, and yields were increased to levels comparable to those obtained from traditional monolayer systems (101). Clark and Hirtenstein (102) optimized culture procedures for cell growth and modified the induction procedure to yield 3×10^4 IU HuIFNβ/10^6 human fibroblasts. This corresponded to 2×10^4 IU HuIFNβ/mg of Cytodex, and the technique could be used to produce 3×10^8 IU HuIFNβ/5 liter culture.

Using Cytodex microcarriers in roller bottles, Kronenberg obtained improved yields (approx. 8-fold) of mouse fibroblast interferon (L. Kronenberg, pers. comm., 103) The cultures used for these experiments are illustrated in Figure 47. Cytodex has also been used to produce human interferon, HuIFNg (G. Alm, pers. comm., 104) Microcarrier culture has enabled the growth of large numbers of human colon carcinoma cells for the production of carcinoembryonic antigen (105) and the production of plasminogen activator from transformed mouse fibroblasts (K. Danø, pers. comm., 106). Further information on the production and purification of specific viruses and cell products from microcarrier cultures can be obtained from Cytiva.

4.2.1 Comparison of microcarriers in different reactors (packed bed or fluidized bed reactor)

Recombinant CHO cells expressing a recombinant protein were cultivated in a packed bed and a fluidized bed reactor and the productivity and functionality of the product compared (Fig 18) (107). Due to better mixing in the fluidized bed, the cells have a better nutrient and oxygen supply (no channeling). Because of the resulting homogeneous environment, a 10-fold increase in productivity could be observed.
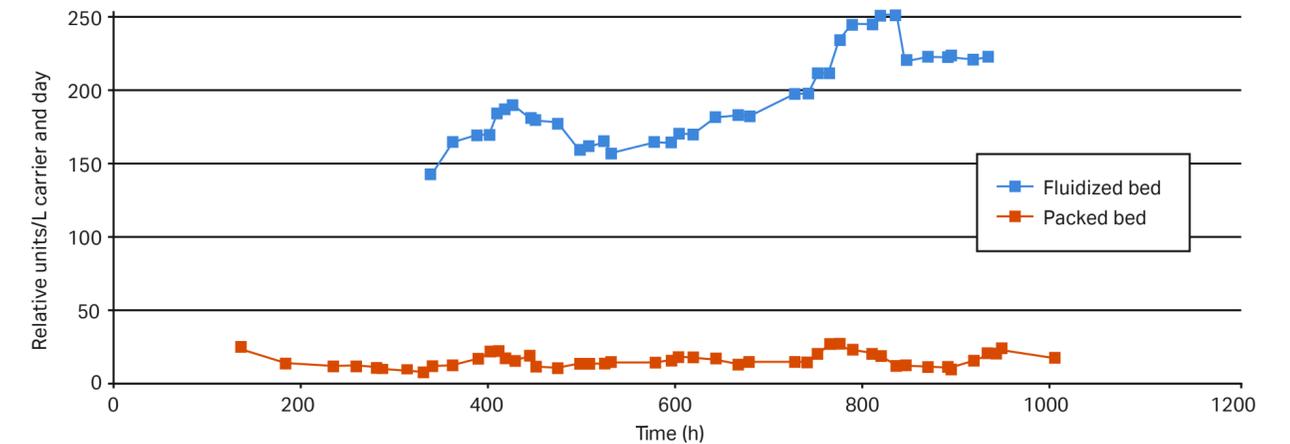
Fig 18. Productivity comparison of packed bed versus fluidized bed.

4.2.2 Long-term production with perfused macroporous microcarriers

The biggest advantage regarding product stability and process economics is the possibility of long-term production with macroporous microcarriers in a perfusion process. The technology of using macroporous microcarriers is one of the best available for running these long processes without breakdowns such as clogging of filters, etc. Cells grow on the macroporous beads until the pores are fully covered. On the outside of the carriers, the cells are sheared off due to bead-to-bead collisions in the stirred tanks or fluidized bed reactors.

Genzyme, Framingham, MA, USA presented information about the development of a long-term microcarrier-based perfusion process for the production of recombinant proteins at the IBC conference "Cell Culture and Upstream Processing", San Diego, December 2003. This process is used for the production of r-protein for enzyme replacement therapies to treat lysosomal storage disorders and runs up to 60 days. The key benefits of this process are: four-fold increase in productivity, decreased cell turnover and lysis rate, higher sialic acid content, cell-free harvest fluid, higher plant throughput and fewer bioreactor starts.

Presuming that the cell line is stable over the entire process (which one has to prove to the regulatory authorities), you should be able to run the perfusion for as long as deemed viable. Figure 19 shows a seven and a half-month fluidized bed run with Cytoline 1. Production was very stable over the entire period.

4.2.3 Process stability of perfusion with macroporous microcarriers

One of the major concerns of perfusion technology is process stability and reproducibility. In one process a recombinant enzyme (enzyme deficient in patients with mucopolysaccharidosis type VI (MPS VI)) is produced. This enzyme (arylsulfatase B) binds to the mannose 6-phosphate receptor of the target cells and is internalized during therapy. This receptor is present on the CHO cells as well. Arylsulfatase B produced by CHO is partly bound again to the CHO cell wall internalized and processed. Because of this, a batch or fed-batch production would result in a more than 50% loss of product. A perfusion process, with its high cell densities and perfusion rate, is essential for economic production of this specific enzyme. The residence time inside the reactor can be reduced to several hours due to perfusion instead of days during batch or fed-batch cultures. Figure 20 shows five runs with this CHO cell type in a Cytopilot fluidized bed with Cytoline microcarriers that demonstrate process stability and reproducibility. Growth rate and final cell density are the same in all runs, despite different inoculation densities or even bead-to-bead transfer inside the reactor.
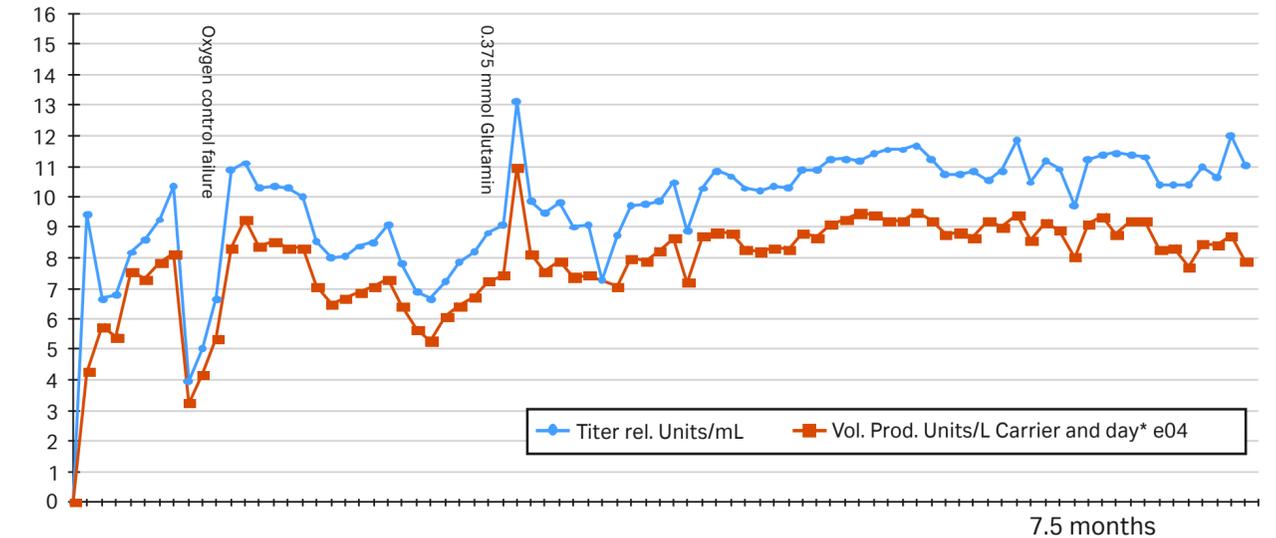
Fig 19. Long-term perfusion process in a Cytopilot fluidized bed with Cytoline microcarriers and CHO cell line. The run lasted 7.5 months with constant productivity over the entire fermentation period. This fermentation was performed at Rentschler Biotechnology.
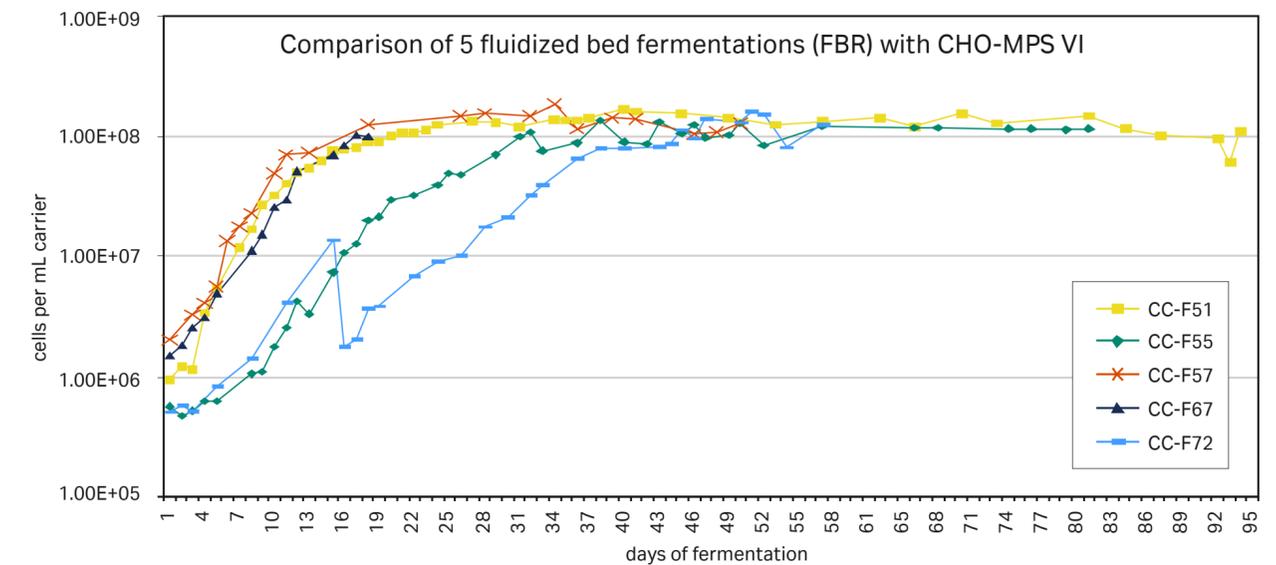
Fig 20. Process stability test. Five Cytopilot runs were performed to demonstrate equal growth rate and the same final cell density. Different inoculation densities were tested and a bead-to-bead transfer made in one of the runs (showed the same growth rate and final cell density).

4.3 Monoclonal antibodies

The increased use of monoclonal antibodies in medical therapy requires cost-efficient production as the dose/patient is normally large. Hirst, senior director of production at Biogen, stated at BioLogic 2001: "150 to 200 products are under development with the launch of around 25 such products likely by the end of 2003. It takes three to five years to build a plant and even longer to validate it, and many companies cannot afford or do not possess the technical knowhow to do this. Also, they cannot rely on contract manufacturers, as these have no vacant capacity, or they are relatively small players on the manufacturing stage.

Looking at Mabs, the annual bulk of one product is estimated to be between 100 and 400 kg (based on patient numbers between 40 000 and 100 000). If 25 new Mabs come onto the market, the total estimated requirement will be 10 000 kg. Calculated with current antibody titers and purification yields of 50% to 70% and a cycle time of 6–13 days, you could expect 170 kg from a 15 000 liter tank. 10 tons of antibody would require 900 000 liters. Current capacity is thought to be 380 000 liters. It is likely that the gap between demand and capacity will actually widen. There will be a search for alternative production facilities, such as transgenic animals and plants. The capacity shortfall is also likely to heighten leverage in investing in process developments.

Therefore, one solution is to increase reactor output. This can be realized by increasing the cell density (increasing productivity). If you add macroporous microcarriers (Cytopore) to your cell suspension and immobilize the cells you are able to increase cell-densities up to ten-fold. Because of the high cell yields you have to run perfusion to support the cells properly. The longer you run the perfused reactor the cheaper the productions process gets.

An alternative to increase the output tremendously or to decrease reactor sizes is to use large-scale fluidized bed technology with Cytoline macroporous carriers.

Using perfusion technology with macroporous microcarrier not only intensifies the process but also increases product quality. Due to the fact that the cells are supported constantly, the product formation inside the cells is kept constant (posttranslational modifications). Perfusion mimics the blood stream inside the body.

Furthermore, perfusion technologies through macroporous microcarriers have no or fewer cells in the harvest stream. This decreased cell load simplifies clarification steps in downstream processing (filtration, STREAMLINE™, Sepharose Big Beads, etc.). Perfusion technology results in higher capacity per reactor volume, higher product quality and higher overall yield (Section 8.6.8).

Packed bed technology was used for production in the past (108). Disadvantages of packed bed technologies are:

No possibility to take samples of cells during process.

Necrotic zones inside the packed bed due to tissue-like structures that enhance channeling of media. Therefore the cells can not be supported with nutrients and oxygen. The overall viability inside the reactor decreases and the formation of desired product is reduced.

In fluidized beds, due to the abrasion of cells (bead-to-bead collisions) on the surface of the macroporous carriers, an aggregation of beads is avoided and the cells can be supported with medium efficiently. With the availability of scalable fluidized bed technology (Cytopilot), high-productivity processes can now be run over prolonged periods to produce active antibodies.

4.3.1 Comparison of a hollow fiber reactor with a fluidized bed reactor

Comparing a hollow fiber reactor with a fluidized bed reactor (109) shows that hollow fiber enables the high level (> 1g/L) production of IgA but with a negative effect on the fraction of active material. The percentage of active fraction was between 30 and 77%. In the fluidized bed reactor cultivated on macroporous carriers, the active fraction was higher than 80% (personal communication). See Section 9.2. for comments on product quality.

4.4 Potential future applications

Applications currently being evaluated are cell expansion of blood cells (Fig 21) via immobilization of hematopoietic stem cells in bioreactors, and the expansion of cytotoxic lymphocytes to generate sufficient cell numbers to be used in cell therapy. In some applications, cells are encapsulated inside a capsule (to protect them from the immune system) and then transplanted (110). Macroporous microcarriers could easily be encapsulated when they are confluent with cells. Epithelial cells grown on Cytodex microcarriers are used for wound healing (burns) (111). Growing cells on (degradable) microcarriers and using the entire cell/carrier complex in transplantation is also discussed in the paper of Schugens *et al.* (112).

Placing Cytodex or Cytopore into the capillaries of a hollow fiber creates the right environment for a three-dimensional tissue such as an artificial liver (Fig 22).
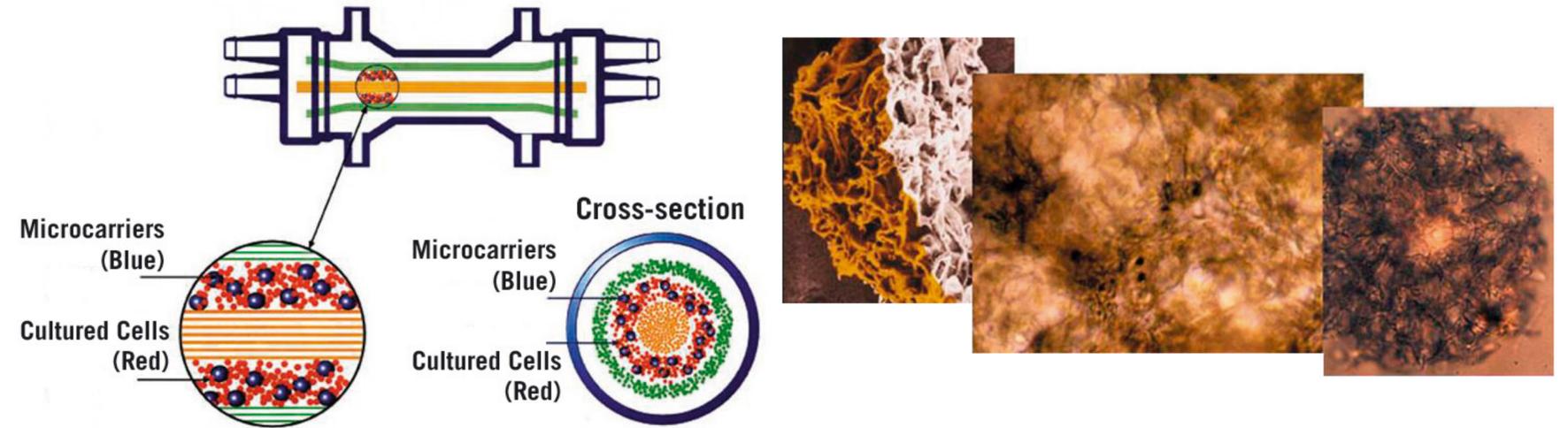
Fig 22. Artificial liver: Hepatocytes were immobilized on Cytopore or Cytodex in a hollow fiber cartridge. The hepatocytes showed the same functionality as in the liver. Picture from Genspan, Seattle, USA.
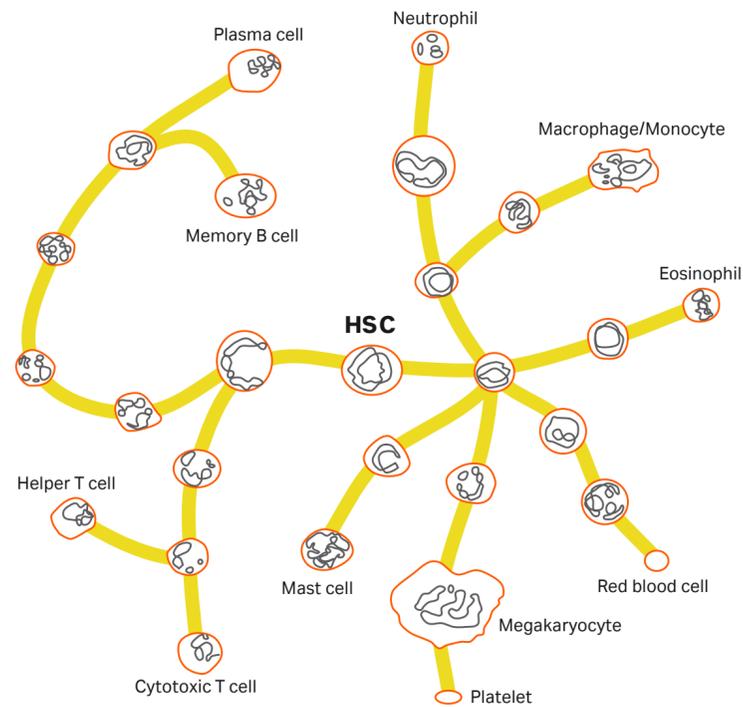
Fig 21. Hematopoietic stem cell (HSC) expansion.

4.5 Further applications

4.5.1 Production of large numbers of cells

A major area of application for microcarrier culture is the production of large numbers of cells. The advantages of the microcarrier system (Section 1.1) can be used to obtain high yields of cells from small culture volumes. Cultures can often be initiated with 10^5 cells/mL or less and at the plateau stage the yield is usually more than 10^6 cells/mL (Fig 23). This high yield of cells per unit culture volume and the large increase in cell number during the culture cycle (10-fold or more) makes microcarrier culture an attractive technique for producing cells from a wide range of culture volumes.

Applications for small culture volumes include situations when only a few cells are available to initiate a culture (e.g., clinical diagnosis, cloned material). Microcarriers can be used to increase the culture surface area in small volumes and at the same time keep the density of cells/mL as high as possible. Maintaining high densities of cells leads to conditioning of the culture medium and stimulation of cell growth. With traditional monolayer techniques for small cultures, it is not possible to achieve a high culture surface area/volume ratio (approx. 4 cm^2/mL in Petri dishes). Microcarrier cultures provide a surface area/volume ratio of approximately 20 cm^2/mL. The increase in culture surface area means that a greater yield of cells is achieved before subculturing is necessary. In clinical diagnosis or the production of cloned material, this technique leads to a reduction in the time required to grow cells for biochemical analyses (113). Figure 24 illustrates the high yield of cells that can be obtained from microcarrier cultures contained in traditional monolayer culture vessels. Microcarrier culture also provides a method for rapid scale-up with a minimum of subculture steps (Fig 18). Scale-up can be through the entire range of culture volumes, and also be achieved in the absence of subculturing steps by using a continuous propagation technique (6). This technique provides sustained periods of exponential growth. Large culture volumes of several liters or more are mainly used for production of viruses or cell products (Section 4.1.1). The yield of cells from large-scale cultures using Cytodex is usually 10^9 cells/liter or more.
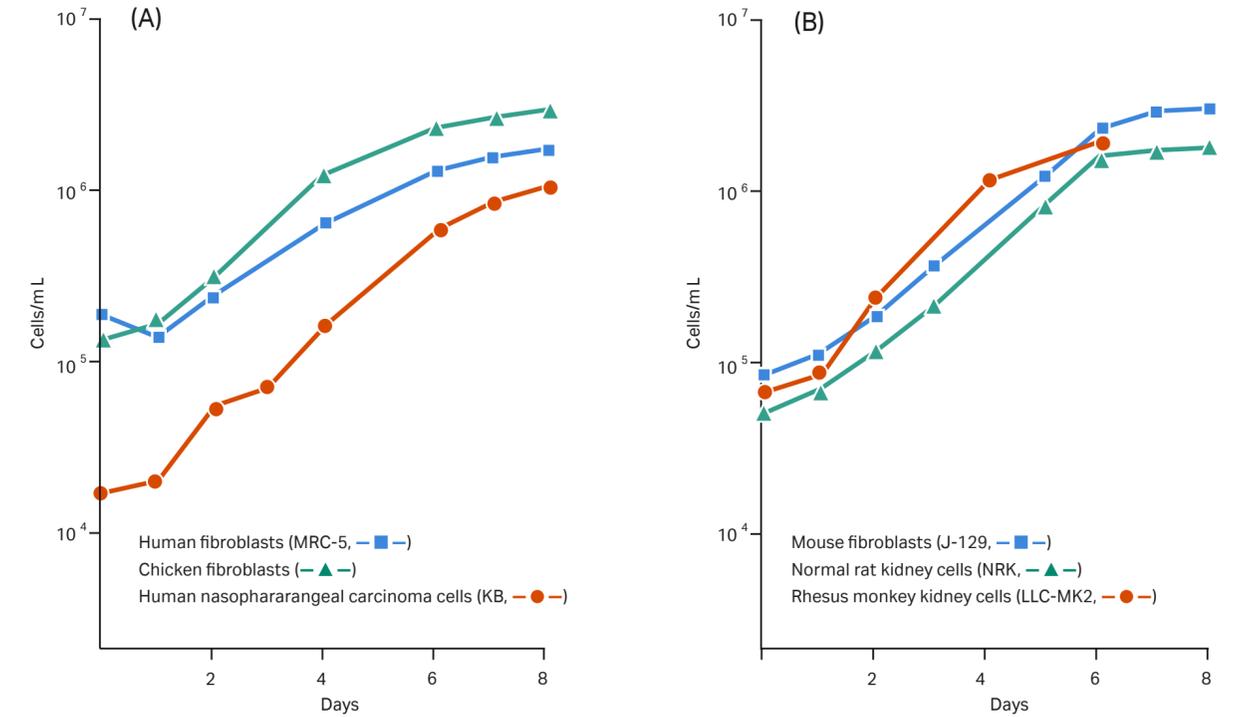
Fig 23. The growth of various types of cells on Cytodex microcarriers in stirred cultures. (Data reproduced by kind permission of S. Toyama, Inst. Virus Res., Kyoto University, Kyoto, Japan.)
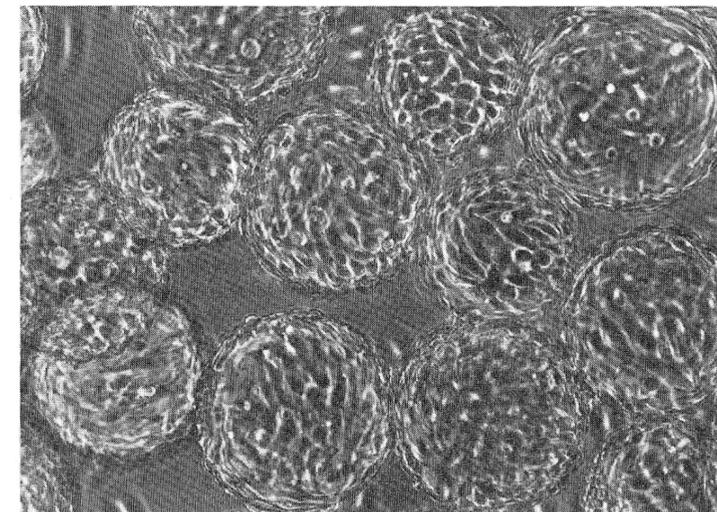
Fig 24. Chinese hamster fibroblasts growing on Cytodex microcarriers contained as a static culture in a Petri dish. (Original photograph by T. Utakoji, Cancer Inst., Japanese Foundation for Cancer Res., Tokyo, Japan, reproduced by kind permission.)

4.5.2 Studies on cell function, metabolism and differentiation

Microcarriers can be used as convenient culture surfaces in many cell biology studies. The ability to culture cells at high densities in a homogeneous culture system provides unique opportunities for studies of cell function, metabolism and differentiation. In addition, microcarriers make it easier to manipulate and observe cells. When compared with traditional monolayer techniques that only provide two-dimensional cultures, the microcarrier system allows very high culture densities and, when confluent microcarriers are packed together, a three-dimensional culture can be achieved.

Cytodex microcarriers are compatible with cell function and differentiation *in vitro*, and a wide variety of studies have been reported. The choice of the most suitable microcarrier is described in Section 4.6 and, for most studies with differentiating systems, Cytodex 3 is the microcarrier of choice.

Several examples serve to illustrate the use of microcarriers in cell biology studies. Pawlowski *et al*. (114) used Cytodex to study the differentiation of chick embryo skeletal muscle cells. Normal myogenesis occurred on the microcarriers, which were also used for microscopy studies (Fig 29 plate 1). After 4 days of culture, 62% of the microcarriers had myotubes with extensive myofibril formation (114). With an even more sensitive cell system, Moser and Stoffels (115) studied the differentiation of newborn rat heart muscle cells. The microcarrier method provided homogeneous and easily manipulated cultures. The heart cells spread and proliferated on the microcarriers and expressed pacemaker membrane properties. Between 20% and 30% of confluent monolayers on the microcarriers exhibited spontaneous beating activity (115).

The release of insulin from fetal rat pancreas islet cells growing on Cytodex has been studied by Bone *et al*. (116, 117). These studies demonstrated that Cytodex is suitable for maintaining highly specialized endocrine cells in culture. The microcarriers provided a method for the uniform suspension culture of functioning pancreas cells and allowed easy manipulation (117). The pancreas cells sustained synthesis and release of insulin during a 7-day growth period on the microcarriers (Fig 29 plate 3), and the release could be modulated by glucose and stimulated with theophylline (117).

Microcarriers have been used in novel culture systems to study the function of differentiated cells. Smith and Vale (118, 119) have developed a super-perfusion column technique to study rat anterior pituitary cells and the modulation of pituitary secretions by gonadotrophins and cocarcinogens (Fig 25). The system provided responsive and well-defined high-density cultures that maintained the ability to secrete hormones for long periods of time. The dissociated pituitary cells attached to the Cytodex microcarriers (Fig 26) and remained responsive to hypothalamic releasing factors (118, 119, Fig 27). Approximately 95% of the cells attached, and the culture system could be used to study transient phenomena and desensitization (118).
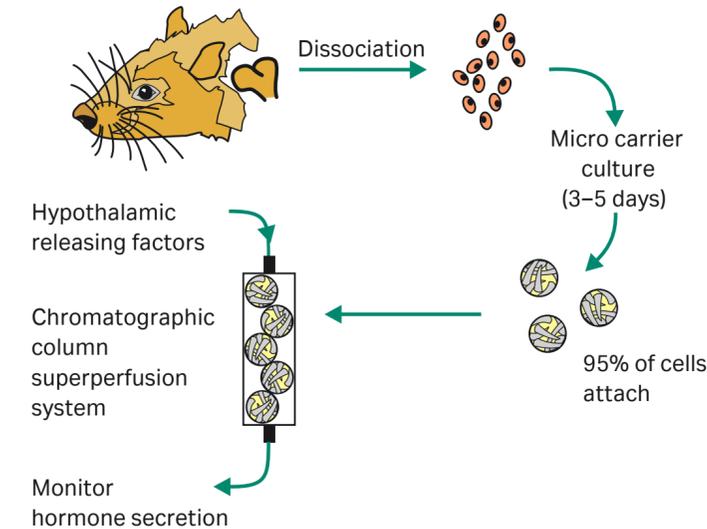
Fig 25. The superperfusion culture of primary rat pituitary cells growing on Cytodex 1 microcarriers. The scheme is based on studies by Smith and Vale (118, 119).
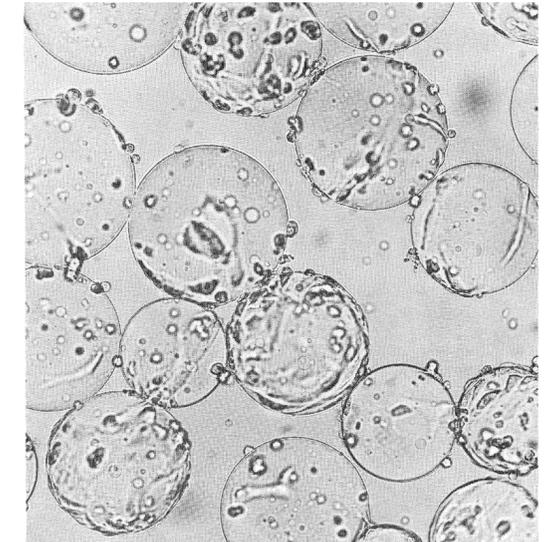
Fig 26. Primary rat interior pituitary cells attached to Cytodex 1 microcarriers and incubated for 5 days after dissociation. (Smith, M.A. and Vale, W.W. Endocrinol. **107**, 1425 (1980) by kind permission of the authors and publisher.)
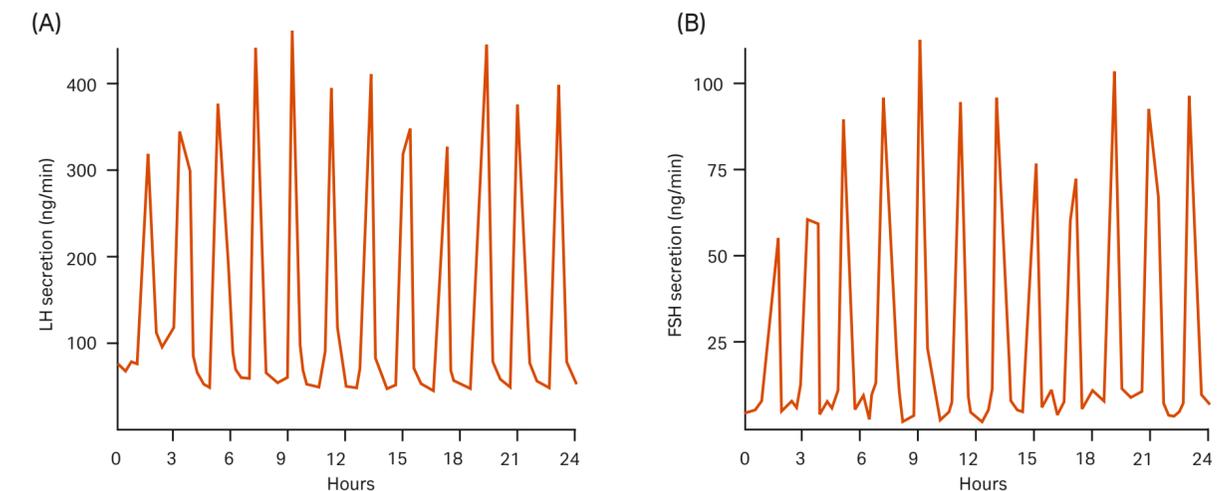
Fig 27. Response of rat anterior pituitary cells growing on Cytodex 1 microcarriers to pulses of gonadotropin-releasing hormone (GnRH). The cells were cultured in the system illustrated in Figure 26 and were exposed to 15 min pulses of 30 nM GnRH every 2 h. Flow rate was 0.2 mL/min and fractions were collected every 20 min.
(A) Secretion of luteinizing hormone (LH).
(B) Secretion of follicle stimulating hormone (FSH).
(Smith, M.A. and Vale, W.W., *Endocrinol*. **108**, 752 (1981) by kind permission of the authors and publisher.)

A variety of other differentiated cells have been studied using Cytodex microcarriers. Ryan *et al.* (120) developed a microcarrier culture system for studying the role of bovine pulmonary endothelial cells and K. Busch (pers. comm., 121) has used Cytodex 3 in studies of endothelial cells from brain capillary and pulmonary artery (Fig 29 plate 2). Porcine thyroid cells cultured on Cytodex exhibited an epithelial morphology and were capable of releasing thyroglobulin (122).

Vosbeck and Roth (123) used microcarrier culture to study the effects of different treatments on intercellular adhesion. Confluent monolayers of cells were cultured on microcarriers and inter-cellular adhesion was examined by studying the binding of ^{32}P-labeled cells to the monolayers (123). Lymphocytes have been grown on microcarriers for studies of stimulation (124). Cytodex 1 alone was not mitogenic for lymphocytes but potentiated stimulation by Con A (Fig 28).

Microcarriers have been used to study the relationship between anchorage, cell density and stimulation of lymphocytes (124), and in studies of animal cell plasma membranes. Lai *et al.* (125) used Cytodex to study the influence of adhesion on the fluidity on Chinese hamster ovary cell plasma membranes. Using electron spin resonance, it was possible to compare cells growing in free suspension culture and attached to microcarriers in suspension culture. Cytodex microcarriers are compatible with spin-labeling and provided a technique whereby cells could be easily transferred and assayed without removal from the culture surface (125).

Microcarrier culture can also be used to isolate plasma membranes with less than 1% contamination from internal membrane markers (126). The procedure is suitable for cells capable of attachment. Cells are first allowed to attach and spread on the microcarriers. Hypotonic lysis is followed by brief sonication to disrupt the cells. The cell debris is then removed, and membranes attached to the microcarriers can be used directly for assays of membrane-associated enzymes (126).

4.5.3 Proteolytic enzyme-free subcultivation and cell transfer

In many studies, it is important to be able to harvest or transfer cultured cells without using proteolytic enzymes or chelating agents, which often alter cell viability and the integrity of the plasma membranes. Microcarriers make it possible to subculture cells or scale-up cultures without using such agents. They also provide convenient surfaces for cell growth, and cells can be transferred from culture vessel to culture vessel or used directly for experiments without having to be removed from the microcarriers. Horst *et al.* (127) and Ryan *et al.* (120) observed that cells could migrate between microcarriers and the surfaces of cell culture flasks or Petri dishes. When Cytodex microcarriers were allowed to settle onto monolayers of mouse fibroblasts, cells migrated onto the microcarriers, which could then be transformed to another culture vessel (127). This method of transferring cells has also been used for bovine pulmonary artery endothelial cells that are normally sensitive to treatment with proteolytic enzymes (120). A variation of this technique is to allow cells to migrate from confluent microcarriers onto new microcarriers. Crespi and Thilly (6) could maintain prolonged periods of exponential growth of CHO cells by diluting confluent microcarrier cultures and adding new microcarriers. Transfer of cells between microcarriers was enhanced by a medium with low Ca^{2+} concentration (6).
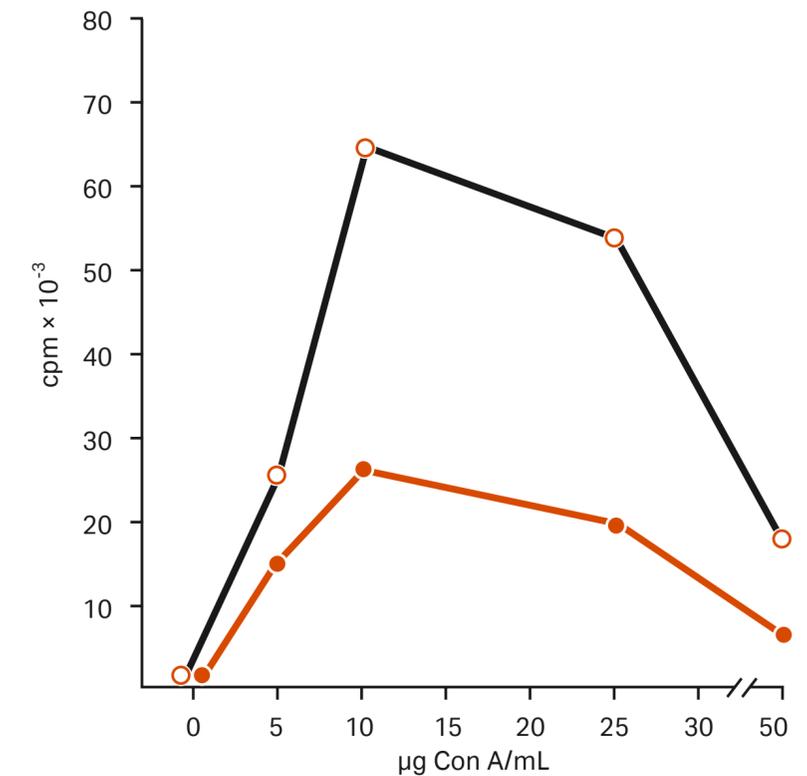
Fig 28. The stimulation of human lymphocytes by Con A in the presence (—○—) or absence (—●—) of Cytodex microcarriers. The microcarriers alone were not mitogenic. (Sundqvist, K. and Wagner, L., Immunology **43**, 573 (1981) by kind permission of the authors and the publisher.)

New microcarriers can also be added to confluent microcarrier cultures during periods of virus production, and Manousos *et al.* (98) used this technique to cause a new wave of cell proliferation and production of oncornavirus. It is also possible to scale up microcarrier cultures of human fibroblasts by allowing confluent microcarriers to settle with new microcarriers. After a few hours, migration of cells results in inoculation of the new microcarriers (P. Talbot, pers. comm., 128).

However, the suitability of this technique is limited by the mobility of cells. Some types of cells, e.g., hepatocytes, exhibit only limited mobility and do not migrate between microcarriers and other culture surfaces. The chance of cell transfer between microcarriers is increased by allowing the culture to remain static for several hours. Occasional stirring should eliminate any tendency for the microcarriers to aggregate.

Microcarriers also have other applications in the transfer of cells. For example, 10 minutes after Cytodex has been incubated with peritoneal fluid, macrophages adhere to it and can be separated from the other peritoneal cells by simple differential sedimentation (H. Slater, pers. comm., 129). The macrophages attached to the microcarrier can then be transferred to other culture vessels for study.

Microcarriers can also be used for cloning cells. Cultures can be inoculated with approximately one cell/microcarrier and, after allowing time for cell attachment, those microcarriers bearing only one cell can be transferred by Pasteur pipette into cloning wells. In this way, microcarriers provide an easily manipulated culture surface. Similarly confluent microcarriers can be embedded in semi-solid medium to form feeder layers.

4.5.4 Microscopy

Cytodex microcarriers can be used as cell culture substrates for a variety of microscopy studies using standard techniques such as scanning electron microscopy (Fig 29, plates 1, 4, 8), transmission electron microscopy (Fig 8) and different types of light microscopy illumination, as well as cytochemistry (Fig 29 plates 2, 3, 5–7, 9). The advantages of using microcarriers for microscopy is that culture substrates are easy to manipulate and cells do not need to be harvested when embedding techniques are used. The dextran-based matrix of Cytodex microcarriers (Section 2.1) can be penetrated by the usual embedding media before sectioning. By using confluent microcarriers, transverse sections through cells adhering to the culture substrate can be readily obtained (Fig 8). Routine samples from microcarrier cultures can be processed for detailed microscopical examination, and cultures containing many coverslips are avoided. Similarly, samples of experimental cultures can be processed for microscopy without requiring large numbers of cells. Details of microscopy with Cytodex microcarriers can be found in Section 8.5.

Plate 1: Top: Chicken embryo skeletal muscle cells (myoblasts) 2 days after inoculation onto Cytodex.
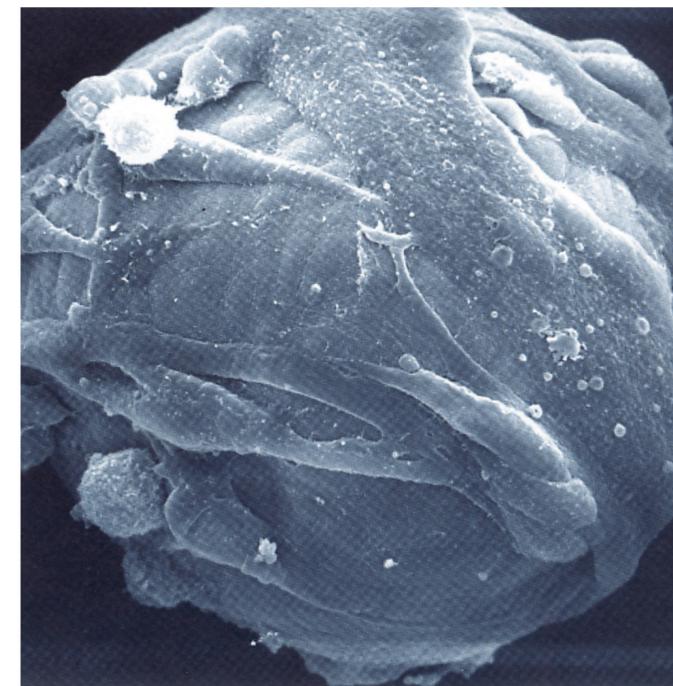
Bottom: Chicken embryo skeletal muscle cells (myoblasts) 7 days after inoculation onto Cytodex. At this stage the microcarrier is confluent and the myoblasts have fused to form myotubes. (Original photographs by Przybylski, R., Pawlowski, R., Loyd, R., Department of Anatomy, School of Medicine, Case Western Reserve University, Cleveland, OH 44106, USA; work supported by the Muscular Dystrophy Association and National Institutes of Health. Reproduced by kind permission.)

Fig 29. Cytodex microcarriers as cell culture substrates. Plate 1.

Plate 2: Top: A confluent monolayer of mouse brain capillary endothelial cells on Cytodex 3.
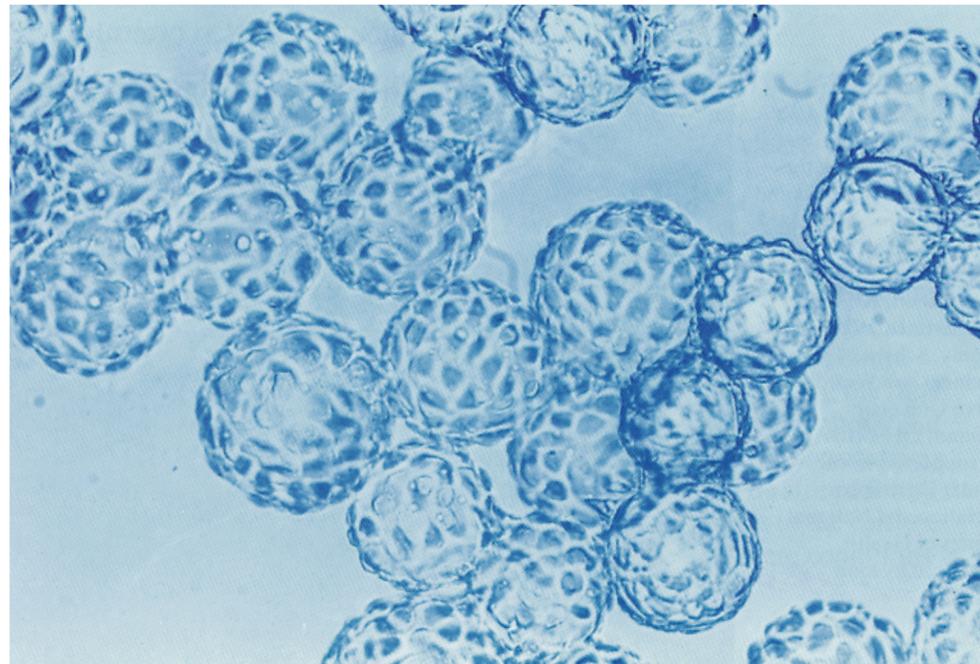
Bottom: Bovine pulmonary artery endothelial cell growing on Cytodex 3. (Original photographs by Busch, C., Department of Pathology, University of Uppsala, Uppsala, Sweden. Reproduced by kind permission.)

Fig 29. continued. Plate 2.
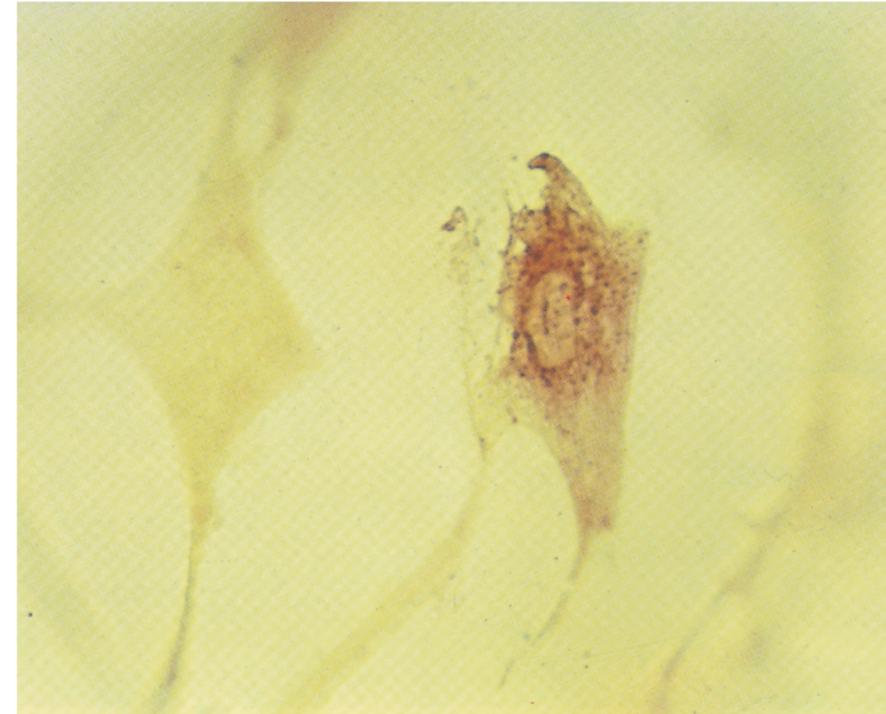
Plate 3: Demonstration of insulin synthesis by immunostaining of fetal rat pancreas cell growing on Cytodex 3. (Original photographs by Bone, A., Swenne, I., Department of Medical Cell Biology, Biomedical Centre, Uppsala, Sweden. Reproduced by kind permission.)
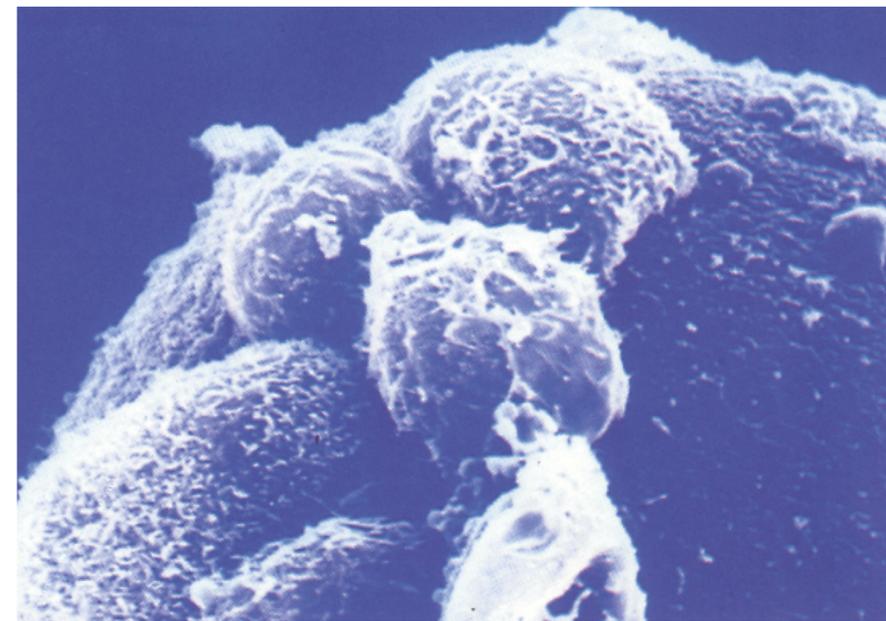
Plate 4: Scanning electron micrograph of human lymphoblastoid cells proliferating on Cytodex. (Original photographs by Christie, W., Gallacher, A., MRC Clinical and Population Cytogenetics Unit, Western General Hospital, Edinburgh EH4 2XU, Scotland. Reproduced by kind permission.)

Fig 29. continued. Plates 3 and 4.
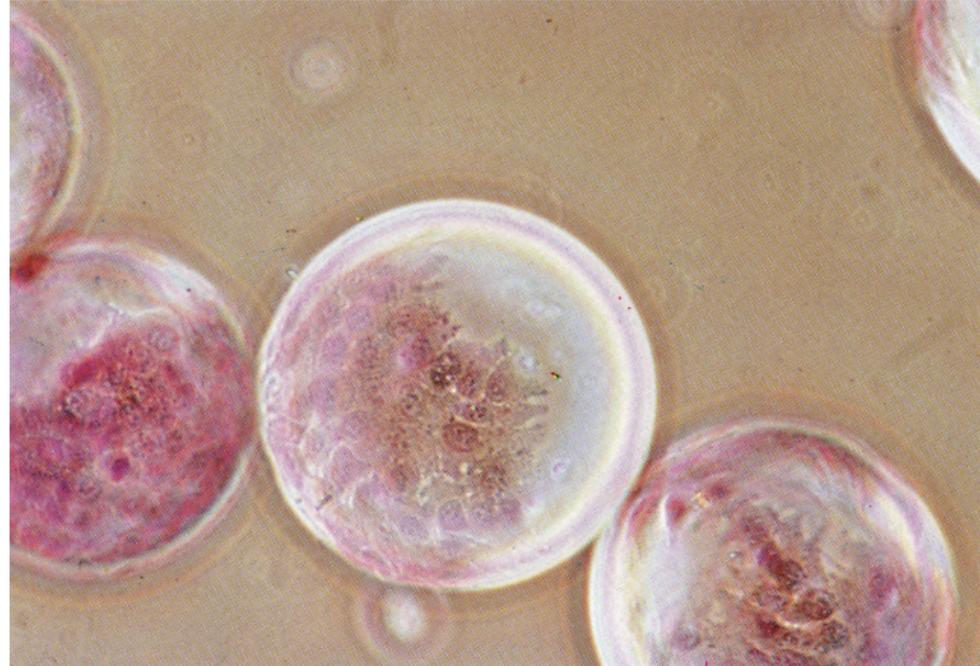
Plate 5: Top: Human cervical carcinoma cells (HeLa) growing on Cytodex microcarriers 3 days after inoculation. Note pronounced epithelial morphology.
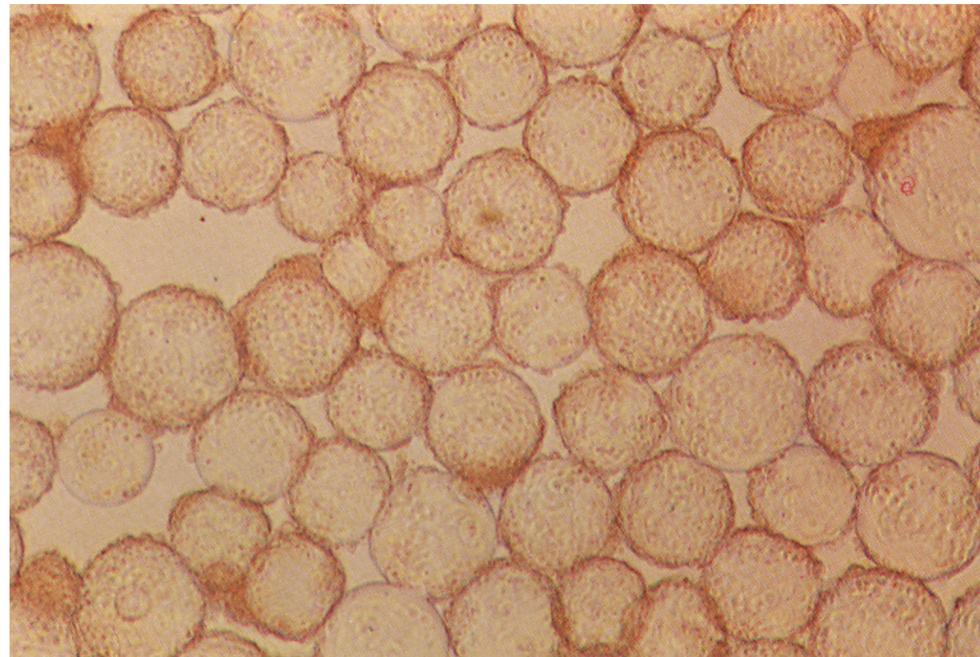
Bottom: Confluent microcarrier culture of human glioma cells. The cells do not exhibit contact inhibition of proliferation and hence multilayers form at confluence. (Original photographs by Pharmacia Fine Chemicals, Uppsala, Sweden. The glioma cells were kindly supplied by K. Nilsson, Wallenberg Laboratory, Uppsala, Sweden.)

Fig 29. continued. Plate 5.
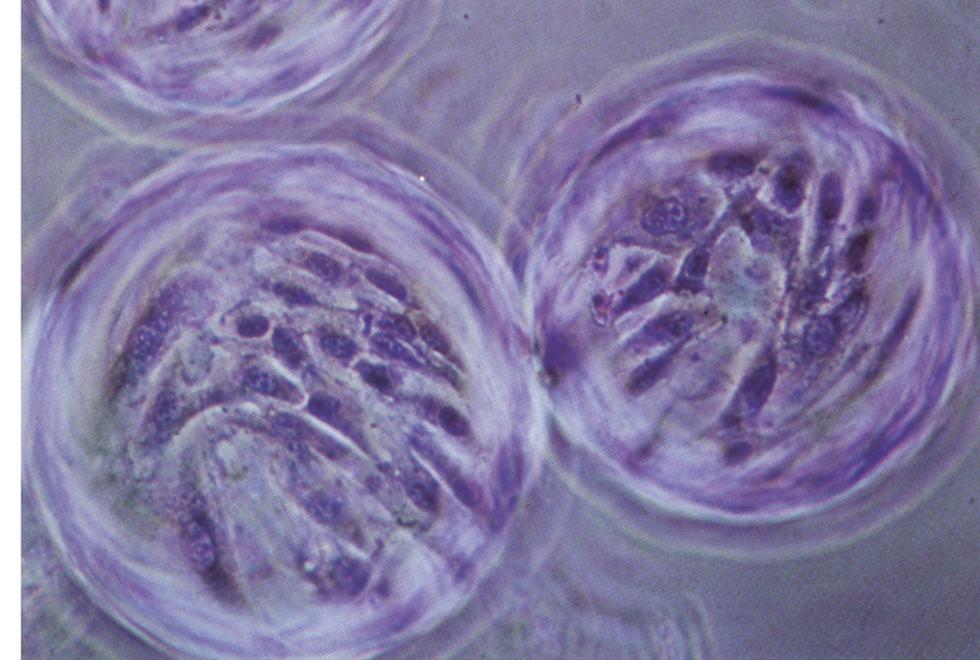
Plate 6: Top: Monolayers of diploid human embryo fibroblasts (MRC-5) on Cytodex microcarriers 7 days after inoculation.
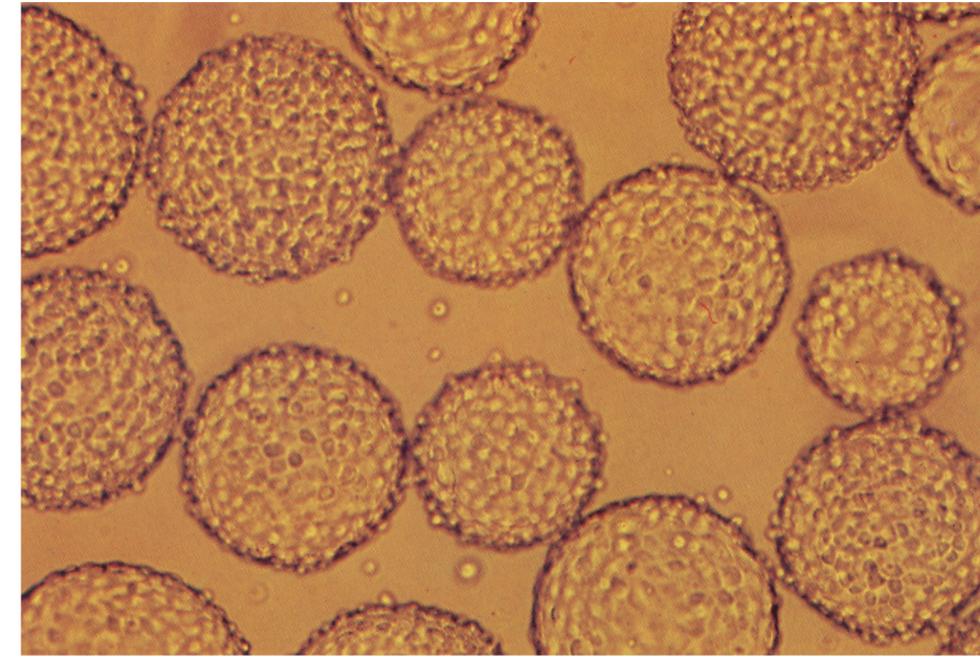
Bottom: A confluent culture of human kidney cells (Flow 4000/Clone 2) on Cytodex 3. (Original photographs by Pharmacia Fine Chemicals, Uppsala, Sweden.)

Fig 29. continued. Plate 6.
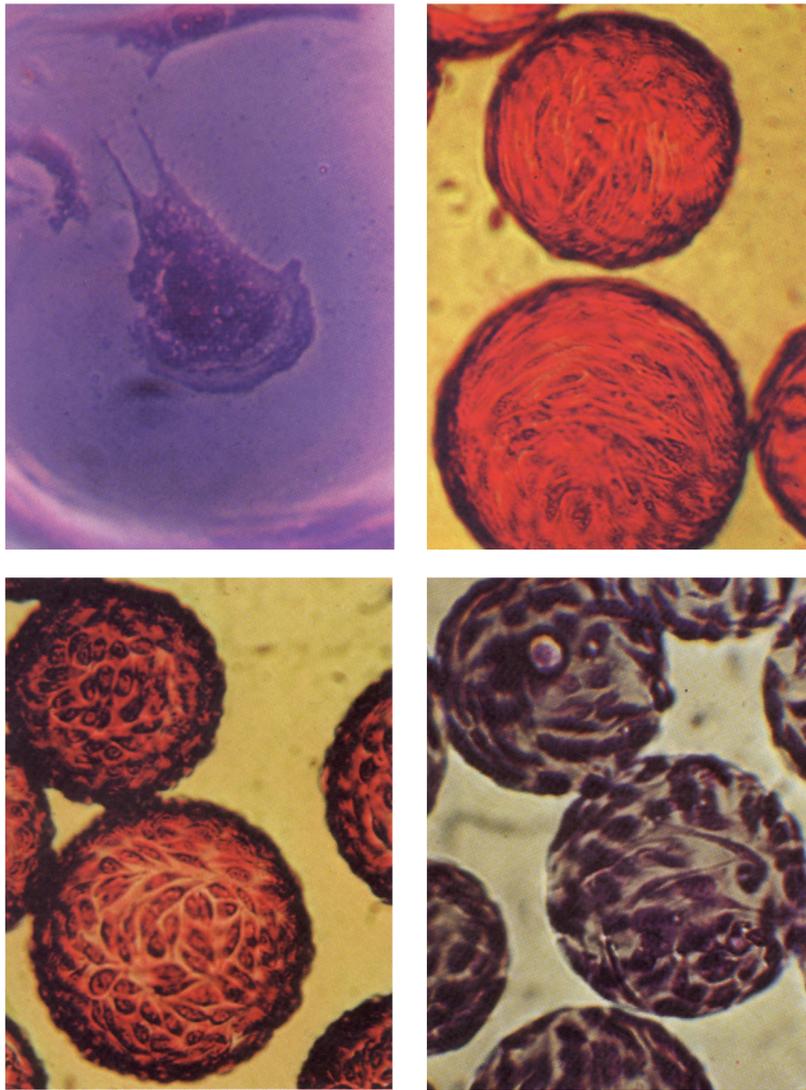
Plate 7: Examples of three common types of cells in microcarrier culture.
Top left: Chicken embryo fibroblasts in motion 24 hours after inoculation.
Top right: Confluent monolayers of chicken embryo fibroblasts.
Bottom left: Chinese hamster ovary cells (CHO) 4 days after inoculation, culture density 7×10^6 cells/mL.
Bottom right: Diploid human foreskin fibroblasts (FS-4). These cells are often used for interferon production in microcarrier cultures (see refs 94, 101). (Original photographs by Tyo, M., Southern Biotech Inc., 3500 E. Fletcher Ave., Suite 321, Tampa, FL 33612, USA. Reproduced by kind permission.)

Fig 29. continued. Plate 7.
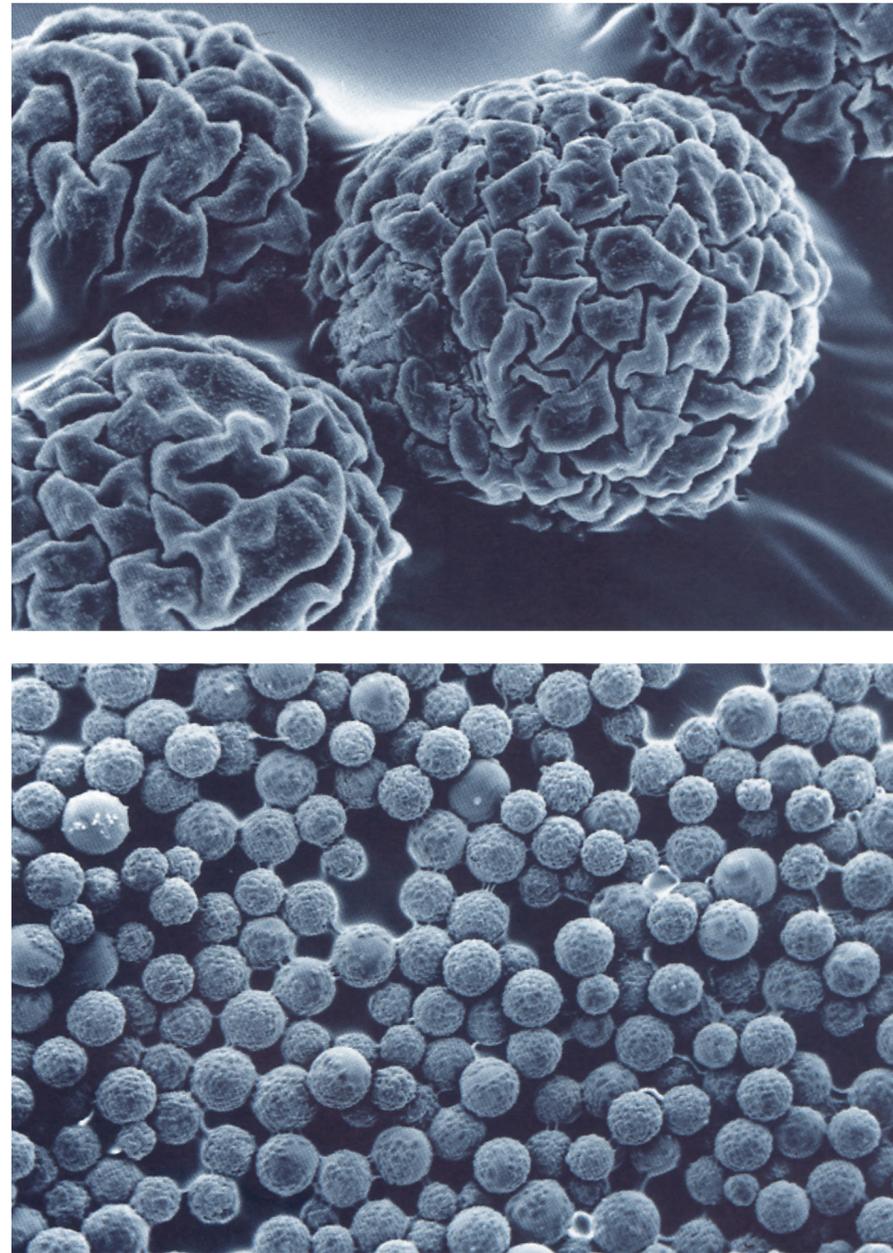
Plate 8: Scanning electron micrographs of pig kidney cells growing on Cytodex in cultures used for the production of foot-and-mouth disease vaccine. (Original photographs by Megnier, B., Tektoff, J., IFFA-Mérieux, 254 rue Marcel Mérieux, Lyon, F-69342 France. Reproduced by kind permission.)

Fig 29. continued. Plate 8.
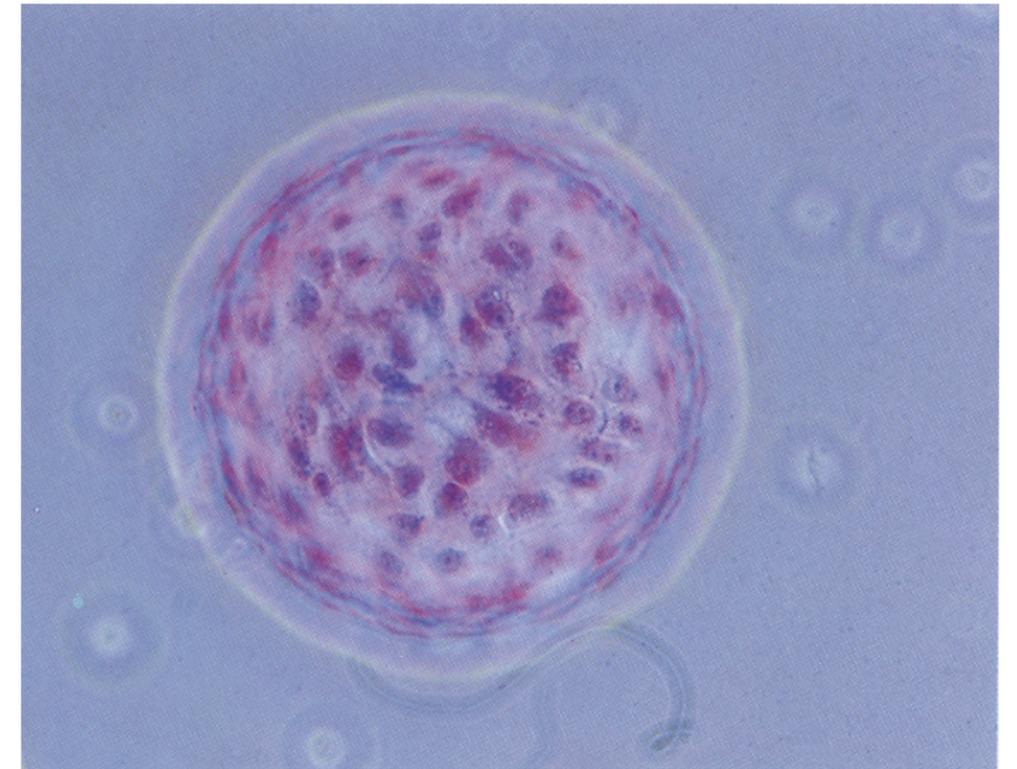
Plate 9: A predominantly epithelial monolayer of primary dog kidney cells growing on Cytodex. (Original photographs by Pharmacia Biotech AB.)

Fig 29. continued. Plate 9.

4.5.5 Harvesting mitotic cells

Microcarrier culture provides an efficient method for harvesting mitotic cells (130, 131). The technique is based on the observation that mitotic cells are attached only weakly to cell culture surfaces and can be detached by shaking (132). The use of monolayer culture vessels for this technique is limited by the small surface area for cell growth. In contrast, microcarriers provide the large surface area necessary for recovering high yields of mitotic cells. Exponential cultures of cells growing on microcarriers in suspension can be treated with mitotic inhibitors (e.g., Colcemid), and by selecting the appropriate stirring speed, mitotic cells can be dislodged and collected in the medium. Mitchell and Wray (130) reported that CHO cells harvested from Cytodex micro-carriers by this method had a mitotic index of up to 88%, a considerable improvement over the mitotic index of 41% obtained when harvesting cells by shaking from a culture flask. Ng *et al.* (131) treated exponential cultures of CHO cells on microcarriers with Colcemid (100 mg/mL) for 2.5 h and then harvested mitotic cells by increasing stirring speed. The increased stirring speed dislodged the mitotic cells, and harvests of more than 4×10^4 mitotic cells/mL of microcarrier culture could be obtained. These cells had a mitotic index of 85% to 95% (131).

The different stages (percentage of detached cells) of cell harvesting for bead-to-bead transfer using trypsinization are shown in Figure 30.
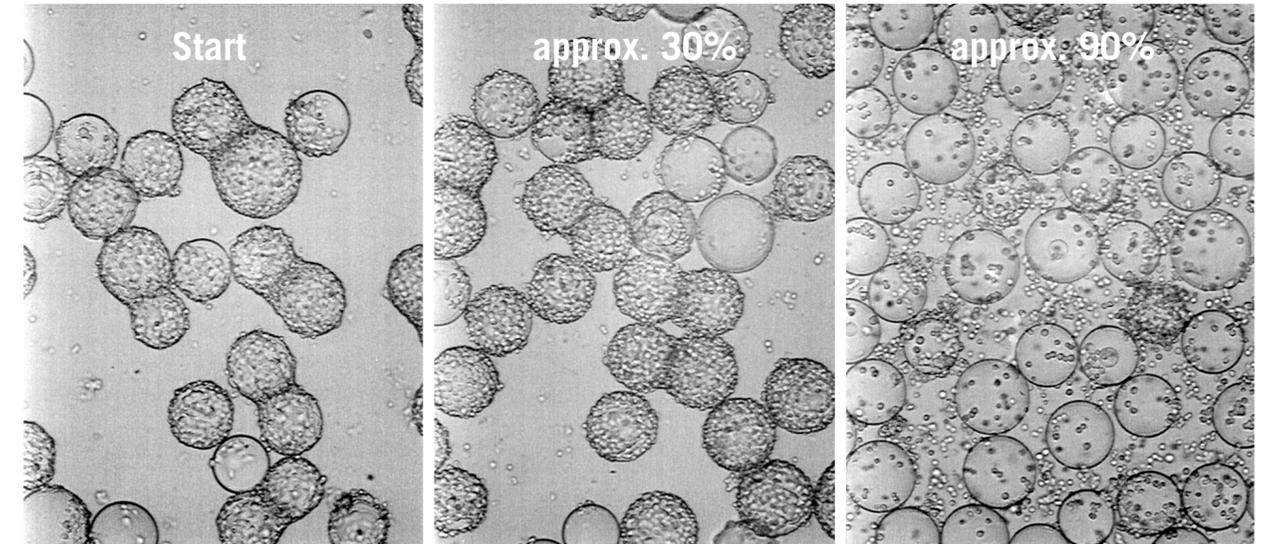
Fig 30. Harvesting cells for scale-up of HEK 293 cells. Different stages of detachment during the trypsinization procedure.

4.6 Which microcarrier to use?

4.6.1 Decision tree

Vaccine Gene Therapy

Lyctic cycle **OR** Nonlyctic cycle

FCS-protein-containing media

Protein-free media

Cytodex 1 **Cytodex 3**

Tissue Engineering

In vivo, ex vivo: all microcarriers possible

Membrane proteins; receptors; cells as product

Adherent cells

Suspension cells

FCS-protein-containing media

Protein-free media

Cytodex 1
Cytodex 3 **Cytopore 1**
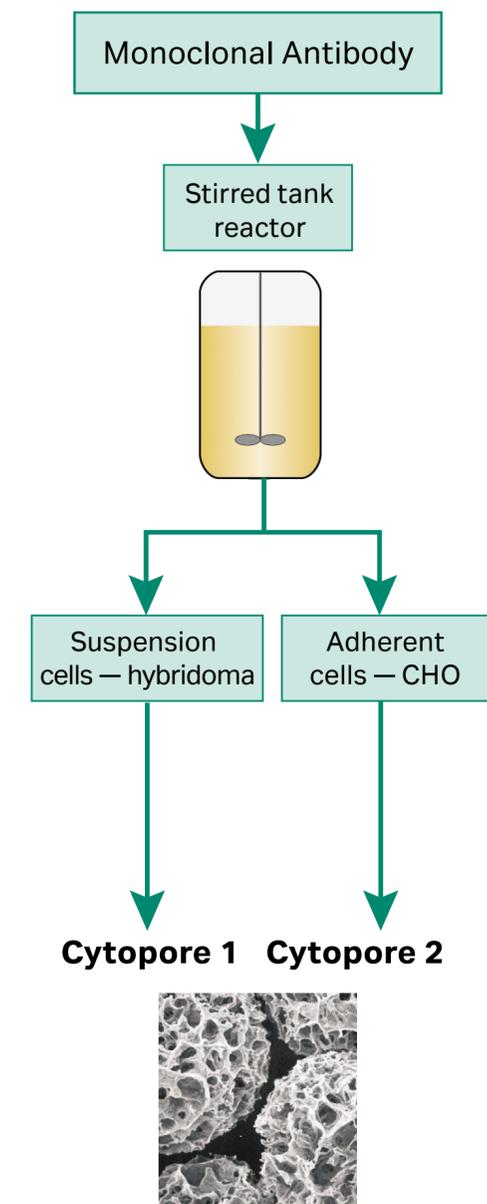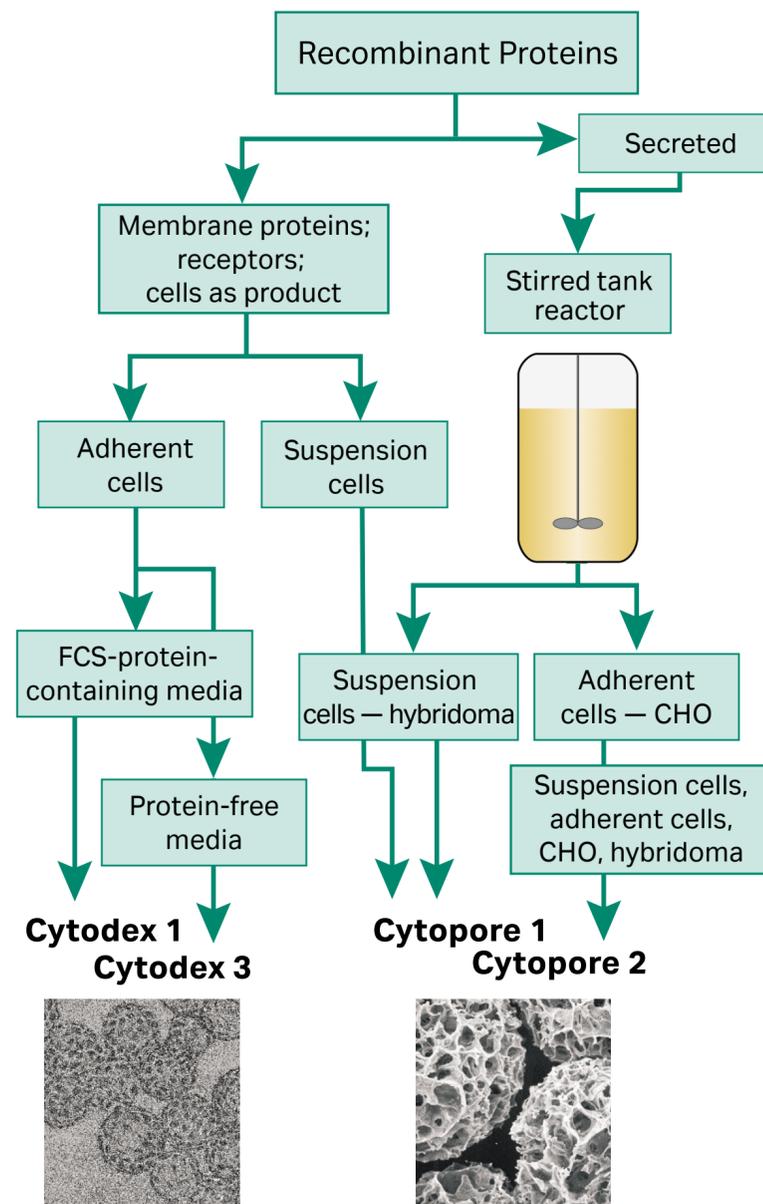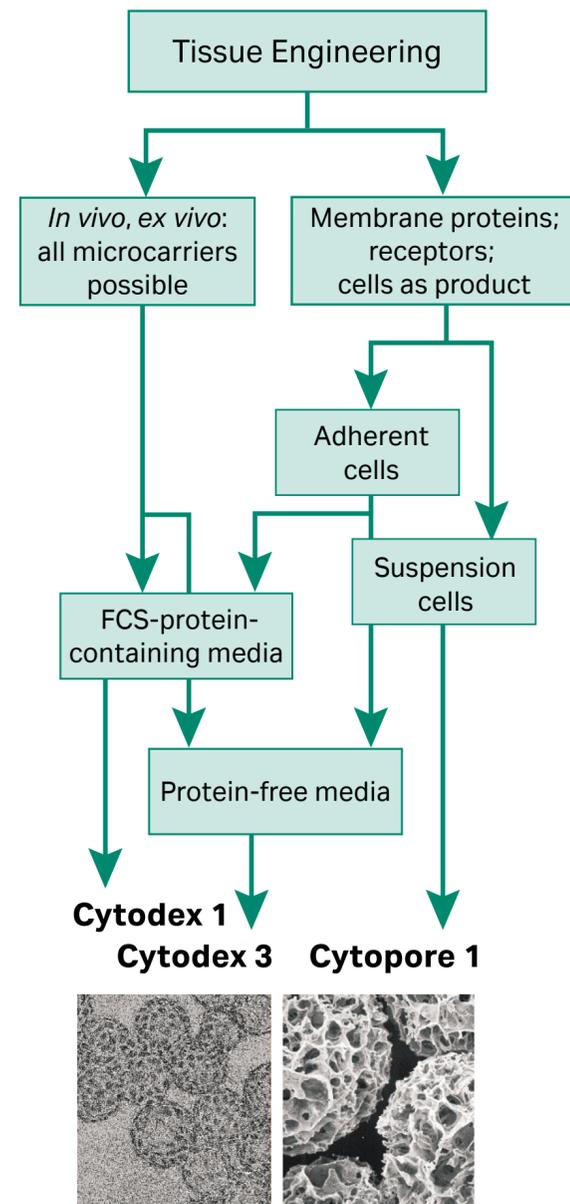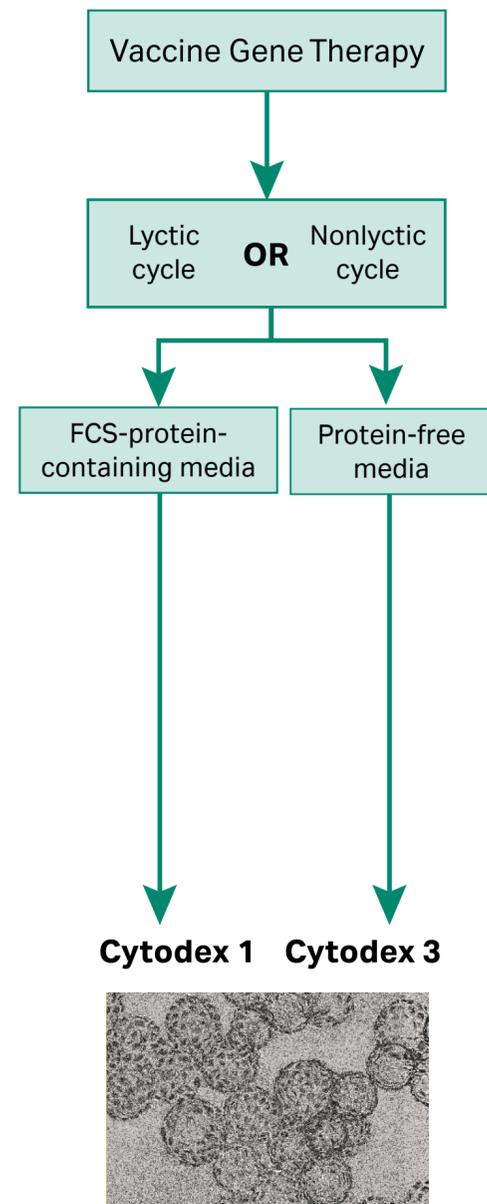
Fig 31. Decision tree for vaccine gene therapy and tissue engineering.

Recombinant Proteins

Secreted

Membrane proteins; receptors; cells as product

Stirred tank reactor

Adherent cells

Suspension cells

FCS-protein-containing media

Suspension cells — hybridoma

Adherent cells — CHO

Protein-free media

Suspension cells, adherent cells, CHO, hybridoma

Cytodex 1
Cytodex 3

Cytopore 1
Cytopore 2

Fig 31B. Decision tree for recombinant proteins.

Monoclonal Antibody

Stirred tank reactor

Suspension cells — hybridoma

Adherent cells — CHO

Cytopore 1 **Cytopore 2**

Fig 31C. Decision tree for monoclonal antibodies.

4.6.2 Further comments on selecting Cytodex microcarriers

Choosing the correct Cytodex microcarrier depends on the type of cell being cultured and the purpose of the culture.

Cells having a low plating efficiency: when a particular type of cell tends to have a low plating efficiency (i.e., less than approx. 15%) use the microcarrier that enables attachment of the maximum number of cells. In some cultures, it may be necessary to use the microcarrier that gives the highest plating efficiency. When culturing cells with fibroblast-like morphology, Cytodex 1 is suitable. For cells having an epithelial-like morphology, plating efficiency is greater with Cytodex 3. Selecting the microcarrier in this way can also result in higher cell yields, simply because the plating efficiency is improved (Fig 35).

Small numbers of cells available for inoculation: it is not always possible to use large quantities of cells to inoculate a microcarrier culture, and it may be necessary to start with a suboptimal number. This situation often arises when working with primary cultures derived from small quantities of tissue. Under such circumstances, use the microcarrier that results in the highest plating efficiency (see above). For primary cultures inoculated with less than approximately five viable cells/microcarrier, Cytodex 3 is recommended. Similarly, if only small numbers of cells from established cell lines with a low plating efficiency (i.e. less than approx. 10%) are available for inoculation, Cytodex 3 is again preferred.

Requirement for improved harvesting and scaling up: when it is necessary to remove cells from the microcarriers with the maximum possible recovery, viability and preservation of membrane integrity, use Cytodex 3 in combination with a proteolytic enzyme for harvesting. Cytodex 3 should also be considered for scaling up cultures when it is important to harvest the maximum number of cells in the best possible condition for inoculating the next culture. Under such conditions, Cytodex 3 should also be considered for cells with fibroblast morphology. The sometimes slower growth of fibroblasts on Cytodex 3 is usually compensated by the improved harvesting and viability of the cells. Hence it is advantageous to use Cytodex 3 for scaling up cultures (for fibroblasts, the final production culture can then use Cytodex 1). The improved harvesting techniques possible with Cytodex 3 are described in Section 8.6.1.

Requirement for removal of medium components or maximum recovery of cell products: in many microcarrier cultures, and as part of production procedures, it is important to wash medium components or cell products from the culture. The amount of protein that binds to Cytodex microcarriers is extremely small. For example, using Cytodex in chromatography experiments, only 4.3% of the total protein present in 100% newborn calf serum adsorbs to Cytodex 1 (1 mL serum/900 mg microcarrier in the column); and less than 1.1% adsorbs to Cytodex 3. The amount of protein adsorbed to the microcarriers from culture medium supplemented with the usual 10% serum is therefore very small.

Table 9 illustrates that it is possible to wash out serum proteins to a much greater extent from cultures with Cytodex 3 than from cultures with Cytodex 1. When it is important to remove medium components easily, or to achieve maximum recovery of cell products, Cytodex 3 is thus preferred.

Table 9. Removal of serum proteins from cultures using Cytodex microcarriers (Results kindly supplied by Dr. A.L. ven Wezel)

Sample*	Cytodex 1 Albumin	IgG	Cytodex 3 Albumin	IgG
Culture fluid	> 1/2000	> 1/125	> 1/2000	> 1/125
First wash	> 1/2000	1/500	> 1/2000	1/500
Second wash	> 1/2000	1/4000	1/32 000	1/16 000
Third wash	1/8000	1/16 000	1/128 000	1/32 000

* Procedure: Secondary monkey kidney cells were grown for 9 days and cultures were washed with Medium 199 without serum (First wash). The cultures were resuspended in Medium 199 without serum and incubated overnight. The wash fluid was removed (Second wash). The culture was resuspended in Medium 199 (without serum) and infected with polio virus. After 3 days culture fluids were harvested (Third wash). The third wash corresponds to the harvest of virus-containing culture fluids when producing vaccines from microcarrier cultures using Cytodex. Proteins were determined by countercurrent electrophoresis. A dilution of 1/32 000 corresponded to 1 mg albumin/mL or 1 mg IgG/mL.

05

Microcarrier products

5.1 Cytodex microcarriers

5.1.1 Cytodex 1

Cytodex 1 microcarriers are based on a cross-linked dextran matrix that is substituted with positively charged N,N-diethylaminoethyl (DEAE) groups to a degree that is optimal for cell growth (Fig 32). The charged groups are found throughout the entire matrix of the microcarrier (Fig 33).

Published procedures for substituting cross-linked dextran with DEAE groups to form microcarriers for cell culture (3, 69, 73, 98) can lead to the formation of a high proportion (up to 35%) of tandem charged groups (133). The chemical reaction conditions used to produce Cytodex 1 microcarriers are controlled so that formation of such tandem groups is minimized (only approx. 15% of groups). The stability and homogeneity of the charged groups is thus enhanced and their possible leakage minimized. Windig *et al*. (134) used pyrolysis mass spectroscopy to examine the possible presence of leaked DEAE dextran in concentrated polio vaccines prepared from microcarrier cultures using Cytodex 1. If leakage of such groups occurred, it was found to be below the limits of detection, i.e., less than 20 ppm (134). The physical characteristics of Cytodex 1 are summarized in Table 12.

The following pack sizes of Cytodex 1 are available: 25 g, 100 g, 500 g, 2.5 kg, and 5 kg. Open and store under dry conditions. Cytodex microcarriers are stable for more than 8 years when stored unopened at room temperature.
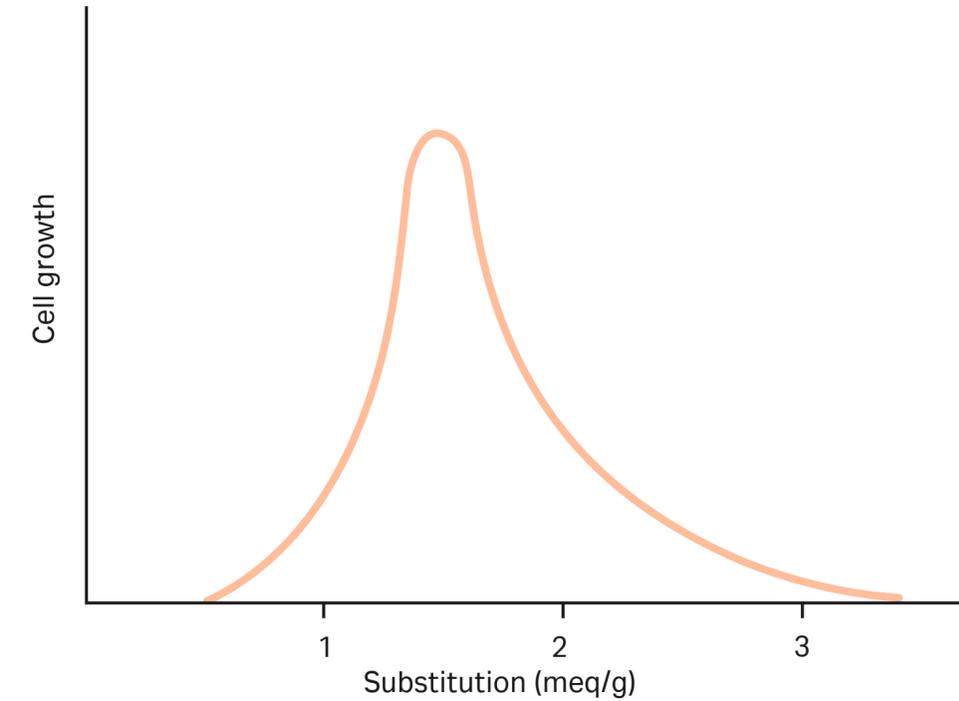
Fig 32. The relationship between the total degree of DEAE substitution of Sephadex G-50 and growth of cells on the resulting microcarriers. The data were pooled from several studies (46, 69, 74 and unpublished work from Pharmacia Fine Chemicals) and concern growth of several strains of human fibroblasts, primary monkey cells and established monkey kidney cell lines in cultures containing 3–5 mg microcarriers/mL. The degree of substitution of DEAE Sephadex A-50 is 3.5 meq/g.
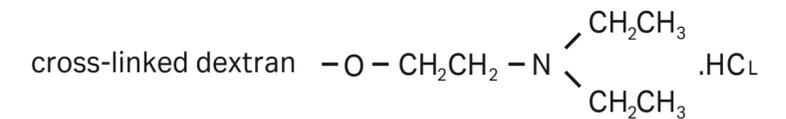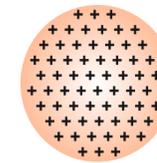
Fig 33. Schematic representation of Cytodex 1 microcarrier. DEAE charges throughout the matrix.

5.1.2 Cytodex 3

Cytodex 3 microcarriers are based on an entirely different principle for microcarrier culture. While most surfaces used in cell culture (e.g., glass, plastic, Cytodex 1) possess a specific density of small charged molecules to promote attachment and growth of cells, certain proteins can also provide a surface for cell growth. The connective tissue protein collagen has proved to be a valuable cell culture substrate. Cytodex 3 microcarriers thus consist of a surface layer of denatured collagen covalently bound to a matrix of cross-linked dextran (Table 10, Fig 34). The amount of denatured collagen bound to the microcarrier matrix is approximately 60 µg/cm^2, which results in maximum cell yields (133). The denatured collagen (Mw 60 000–200 000) is derived from pig skin type I collagen that has been extracted and denatured by acid treatment, concentrated and purified by an ion exchange step, and steam sterilized before being coupled to the matrix.

These microcarriers combine the advantages of collagen-coated culture surfaces with the advantages of microcarrier culture. Cytodex 3 can also be used as a general-purpose collagen-coated cell culture substrate. Most normal epithelial cells will attach more efficiently to collagen than to other surfaces. Consequently, collagen-coated culture surfaces are used frequently for establishing primary cultures and for growing cells that are normally difficult to grow in culture (135, 136). Collagen-coated surfaces are valuable because they permit differentiation of cells *in vitro* even at very sparse or colonial culture densities (136, 137). Such surfaces are also advantageous when culturing for extended periods, since they delay the detachment of the cell sheet that eventually occurs in long-term mass culture on uncoated surfaces (138).

A variety of different types of cells are routinely cultured on collagen-coated surfaces and include hepatocytes, fibroblasts, chondrocytes, epidermal cells, myoblasts and mammary epithelial cells (139). Differentiation of myoblasts at sparse densities *in vitro* depends on the presence of collagen bound to the culture surface (137, 138). Myoblasts attach and spread more satisfactorily on collagen than on standard cell culture surfaces (138), and growth is also stimulated (140). Hepatocytes can be cultured more successfully on collagen surfaces. The collagen permits freshly isolated hepatocytes to attach with maximum efficiency, and spreading is more rapid than on any other cell culture surface (141). Since exogenous fibronectin is not required for attachment of hepatocytes to collagen (142), this surface is the most suitable for culture of hepatocytes in protein-free media (141).

Table 10. Some physical characteristics of Cytodex microcarriers

	Cytodex 1	Cytodex 3
Density* (g/mL)	1.03	1.04
Size* d_{50} (µm)	180	175
d_{5-95} (µm)	131–220	133–215
Approx. area* (cm^2/g dry weight)	4400	2700
Approx. no. microcarriers/g dry weight	6.8×10^6	4.0×10^6
Swelling* (mL/g dry weight)	18	14

Size is based on diameter at 50% of the volume of a sample of microcarriers (d_{50}), or the range between the diameter at 5% and 95% of the volume of a sample of microcarriers (d_{5-95}). Thus size is calculated from cumulative volume distributions.
* In 0.9% NaCl
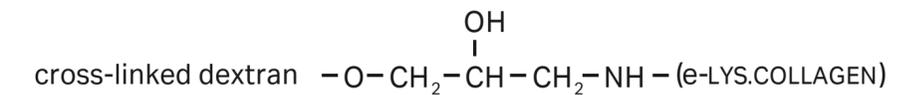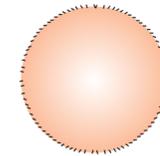
Fig 34. Schematic representation of Cytodex 3.

A collagen culture substrate also allows for more extended studies of hepatocytes *in vitro* with improved retention of differentiated function (143, 144). Folkman *et al*. (145) have described the culture of capillary endothelial cells from a variety of sources on collagen, and excellent growth of endothelial cells on Cytodex 3 has been demonstrated (Fig 29 plate 2). Geppert *et al*. (146) reported that there was better maintenance of differentiated alveolar type II cell function when using collagen as the culture substrate. Surface-bound collagen can also be used to culture fibroblasts (147, 148), and Cereijido *et al*. (149) described cross-linked collagen for culturing the epithelial kidney cell line, MDCK. Secondary bovine embryo kidney cells have a higher plating efficiency when grown on Cytodex 3 than when grown on Cytodex 1. This improvement in plating efficiency leads to higher cell yields (Fig 35).

Cytodex 3 microcarriers are coated with denatured type I collagen as this is the most generally useful form of collagen for cell culture. Although certain types of cells may show specificity for attachment to a particular form of native collagen, this is much less apparent when denatured collagen is used. Adsorption of the attachment glycoprotein fibronectin to the culture surface is known to be important in the adhesion of many cells (Section 2.1) and fibronectin is believed to be also involved in the attachment of many cells to both native and denatured forms of collagen (150). Fibronectin binds equally well to all types of collagen (151) but shows a preference for denatured forms (139, 152, 153) and binds more rapidly to Sephadex beads coated with denatured collagen than to beads coated with native collagen (154).

In the past, a major difficulty with using collagen-coated surfaces has been the rapid loss of collagen from the surface. Kleinman *et al*. (150) observed that up to 40% of the collagen is lost from coated Petri dishes within 90 minutes of adding culture medium. Floating collagen gels (136) have greater retention of collagen but are difficult to prepare and often shrink during the culture period (144). Because the denatured collagen is cross-linked to the microcarrier matrix, the problems of shrinkage, cracking and leakage that are found with standard techniques for coating cell culture surfaces are avoided with Cytodex 3. A further advantage of Cytodex 3 as a general-purpose collagen-coated culture surface is that because of their porous matrix, nutrients have access to the basal cell surfaces. Such access is not possible with collagen-coated plastic or glass surfaces. The denatured collagen layer on Cytodex 3 is susceptible to digestion by a variety of proteases, including trypsin and collagenase (Section 8.6.1.8) and provides unique opportunities for removing cells from the microcarriers. The physical characteristics of Cytodex 3 are summarized in Table 12.

The following pack sizes of Cytodex 3 are available: 10 g, 100 g, 500 g, 2.5 kg, and 5 kg. Open and store under dry conditions. Cytodex microcarriers are stable for more than 8 years when stored unopened at room temperature.
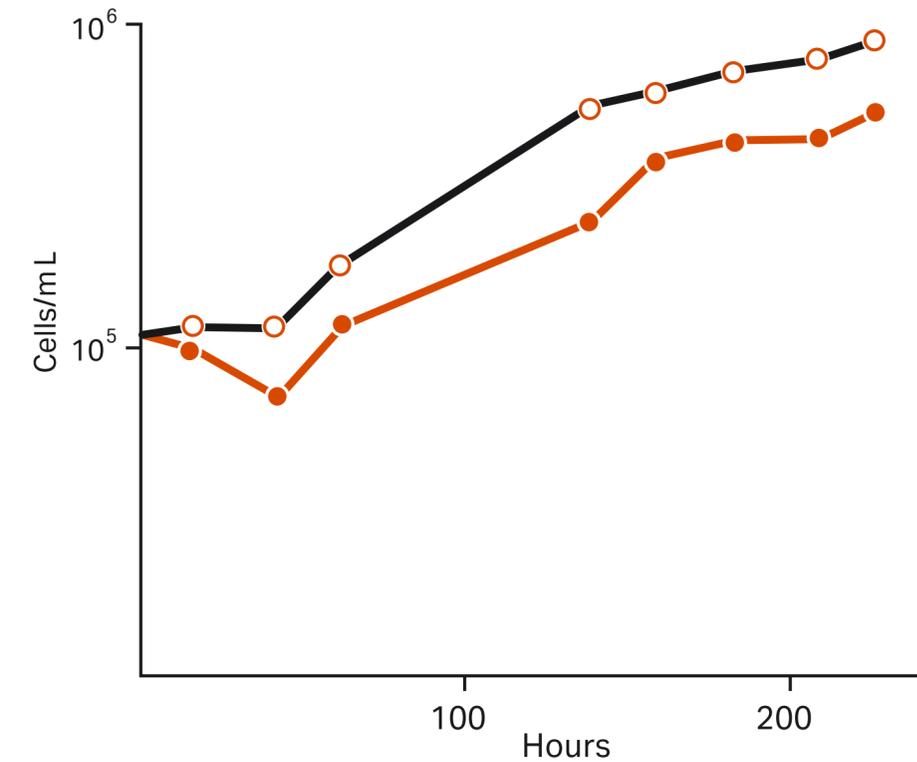
Fig 35. The growth of secondary bovine embryo kidney cells on Cytodex 1 (—●—) and Cytodex 3 (—○—) microcarriers. The cells were a mixed population with predominantly epithelial morphology. (Gebb, Ch., Clark, J.M., Hirtenstein, M.D. *et al*., *Adv. Exp. Med.* **172**, 151–167 (1984), by kind permission of the authors and the publisher.)

5.1.3 Cell types cultured on Cytodex microcarriers

Cell types successfully cultured on microcarriers are listed in Table 11. Virtually all classes of cultured animal cells are represented. Cytodex microcarriers are cell culture surfaces of general applicability, and cells capable of attaching *in vitro* will be able to attach to them. Cytodex 3 also provides an improved culture surface for many types of cells that attach or function poorly on glass or plastic culture surfaces.

Table 11. Tissue-specific list of cell types cultured on Cytodex microcarriers

Tissue	Cell line
Adrenal	Mouse cortex tumor — Y-1
Amnion	Human — WISH
Amniotic cells	Human amniotic fluid
Bone Marrow	Human — Detroit 6 Human — Detroit 38
Carcinoma	Human nasal — RPMI 2650 Human larynx — HEp 2 Human oral — KB Human cervical — HeLa Human colon Human thyroid
Conjunctiva	Human — Chang "D"
Cornea	Rabbit — SIRC
Endothelium	Rabbit coronary endothelium Human coronary endothelium Mouse brain capillary endothelium Bovine pulmonary artery endothelium
Epithelium	Human — NITC 2544
Fibroblast	Human foreskin — FS-4 Human foreskin—Detroit 532 Human — SV40-transformed WI-38 Mouse — SC-1, 3T3, 3T6, L-cells, L-929, A9 Mouse — transformed Mouse — embryo Chicken — embryo Human — embryo Rat — embryo Rabbit — embryo Human — Xeroderma pigmentosum Muntjac — adult skin
Fibrosarcoma	Human — HT 1080 Mouse

Tissue	Cell line
Fish	Rainbow trout gonad — RTG Fat head minnow — FHM Carp eptihelioma — EPC
Glial	Rat
Glial tumor	Rat — C6
Glioma	Human
Heart	Human atrial appendage — Girardi heart
Hepatoma	Rat — HTC, Morris MH1C1
Insect	Drosophila Spodoptera Trichoplusia
Kidney	Human embryo Human embryo — Flow 4000, L-132 Bovine embryo — MDBK Monkey — primary Dog — primary, MDCK, transformed Rabbit — primary, NZ white, LLC-RK RK-13 Rat — NRK, transformed Pig — PK-15, IBR Syrian hamster — HaK, BHK, transformed Potroo — Pt-k-1 Rhesus monkey — LLC-MK2 African Green monkey — Vero, CV-1 BSC-1, BGM, GL-V3
Leukemia	Human monocytic — J111
Liver	Human primary hepatocytes Rat primary hepatocytes Chimpanzee Human — Chang liver

Tissue	Cell line
Lung	Chinese hamster — Don Chimpanzee embryo — CR-1 Human embryo — L-132, MRC-5, MRC-9, WI-38, IMR-90, Flow 2002, HEL 299 Cat embryo Bat — Tb 1 Lu Mink — Mv 1 Lu
Lymphoid	Human — lymphoblastoid Human — lymphocytes
Macrophage	Mouse — peritoneal, peripheral blood Rat — peritoneal Human — peripheral blood Mouse — P388D1
Melanoma	Human Mouse
Muscle	Chicken myoblasts Rat muscle-derived fibroblasts
Neuroblastoma	Mouse — Neuro-2a
Osteosarcoma	Human
Ovary	Chinese hamster — CHO
Pancreas	Rat
Pituitary	Rat Bovine
Rhabdomyosarcoma	Human — RD
Synovial fluid	Human — McCoy
Thyroid	Pig

Mammalian, avian, fish and insect cells have been cultured on Cytodex. These are of wide histotypic origin and include primary cells, diploid cell strains and established or transformed cell lines. Hybrid cell lines and cells of tumor origin can be cultured on Cytodex microcarriers (Fig 36). Selection of the most suitable microcarrier depends on cell type and application (Section 4.6). Examples of cells growing on Cytodex microcarriers are shown in Figure 29 plates 1–9 and other figures throughout this book.

The only types of cells that have proven difficult to grow on microcarriers in stirred cultures have been some of lymphoid origin. Such cells attach only weakly to cell culture surfaces and are dislodged from the microcarriers if the culture is stirred too vigorously. Lymphocytes and lymphoblastoid cells have been successfully cultured on Cytodex microcarriers (Fig 29 plate 4; G. Alm, pers. comm., 104) and good attachment can be achieved when correct procedures are followed (Section 8.4.2, 8.4.3). Cytodex is not mitogenic in cultures of lymphocytes (124).

Anchorage-independent cells can be grown on Cytodex. Although these cells can be grown in free suspension culture, there are often distinct advantages to using microcarriers and still retaining the benefits of suspension techniques.

- Higher culture densities can often be achieved with microcarriers, and culture productivity can be increased

- Separating cells from the liquid phase of the culture is simpler when using microcarriers — long sedimentation times, complicated filters or centrifuges are not needed

- Cultures containing microcarriers are more homogeneous. By allowing the microcarriers to settle, dead cells and debris can be removed in the supernatant fluid. The culture can therefore be enriched for living cells.

- Cells growing on culture surfaces often use medium more efficiently than in free suspension (155). Microcarriers reduce the medium requirements of suspension cultures.

- The yield of many strains of virus is greater when the cell substrate is grown attached to a culture surface. Some viruses (e.g., Herpes) grow poorly in free suspension systems. In general, cells have the same growth kinetics on Cytodex microcarriers as they do on standard glass or plastic culture surfaces (Fig 14, Fig 71). Providing culture conditions are optimal (Section 8.4.2, 8.4.3), most cells will retain their characteristic morphology, population doubling-time and saturation density when growing on Cytodex (Fig 14). Cells that grow with a pronounced fibroblast-like morphology may have reduced saturation density on microcarriers since the spherical growth surface cannot be completely covered by the parallel array of cells.
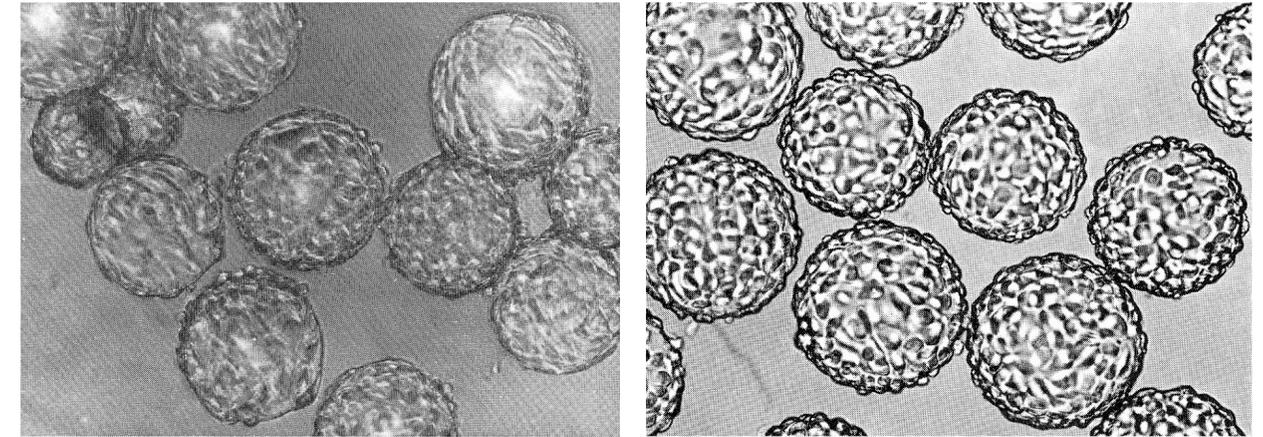
Fig 36. Left: Human-mouse hybrid cells growing on Cytodex microcarriers. The parental cells for the hybrid were human lymphocytes and HGRT mouse cells. (Original photograph by B. Winchester, Queen Elizabeth College, London, UK, reproduced by kind permission.)
Right: Human osteosarcoma cells cultured on Cytodex microcarriers. (Original photograph by B. Westermark and J. Pontén. Wallenberg Lab., Uppsala, Sweden, reproduced by kind permission.)

5.2 Cytopore microcarriers

The major cell culture application areas for Cytopore are production of recombinant products and monoclonal antibodies from CHO, BHK, NSO and hybridoma cells. Due to its macroporosity, the culture of not only anchorage-dependent cells but also cells in suspension or semi-adherent cells is possible.

Cytopore is designed for stirred tank reactors. The physical properties of Cytopore are very similar to those of Cytodex. The design of the stirred tank reactor, impeller and retention device can be the same for both microcarriers.

Cytopore is composed of cross-linked cellulose (cotton linter). With a pore size averaging 30 µm, its porosity is more than 90% (Fig 37). Like Cytodex this microcarrier is also microporous and hydrophilic due to the introduction of DEAE groups on the cellulose.

A certificate of analysis is available for each batch upon request. Each batch of Cytopore is tested for particle size distribution, buoyant density, loss on drying, heavy metals and microbial contamination. In addition, each batch is function tested by culturing CHO-K1 for 150 hours to ensure that the microcarriers support high-density growth. All these methods are described in the Regulatory Support File, which is available under secrecy agreement.

The features of Cytopore are:

1. Optimized surface characteristics for efficient attachment and spreading of cells.

2. Optimized size, density and macroporosity to facilitate even suspension/fluidization and give good growth and high yields for a wide variety of cells.

3. Biologically inert matrix provides a strong non-rigid substrate for stirred microcarrier cultures.

4. Microcarriers are macroporous, which protects the cells from shear forces and allows the cells to create a micro-environment.

Cytopore microcarriers can be used as cell culture substrates for a variety of microscopy studies using standard techniques such as scanning electron microscopy, transmission electron microscopy and confocal laser scanning, as well as different types of light microscopy. Any of the usual fixation and staining procedures can be used with Cytopore. The most suitable procedures for staining cells grown on Cytopore microcarriers use either Giemsa stain or Harris' hematoxylin. The latter stain can be used when better nuclear detail is required.

Using microcarriers for more than one culture/harvest cycle is not recommended. Normally cleaning validation is much more costly than using the microcarriers only once. Then you are guaranteed the same surface property.

This microcarrier has a high rigidity and is very stable at both high and low pH. It can withstand 10 autoclavings at 130°C for 30 min without loss of function.
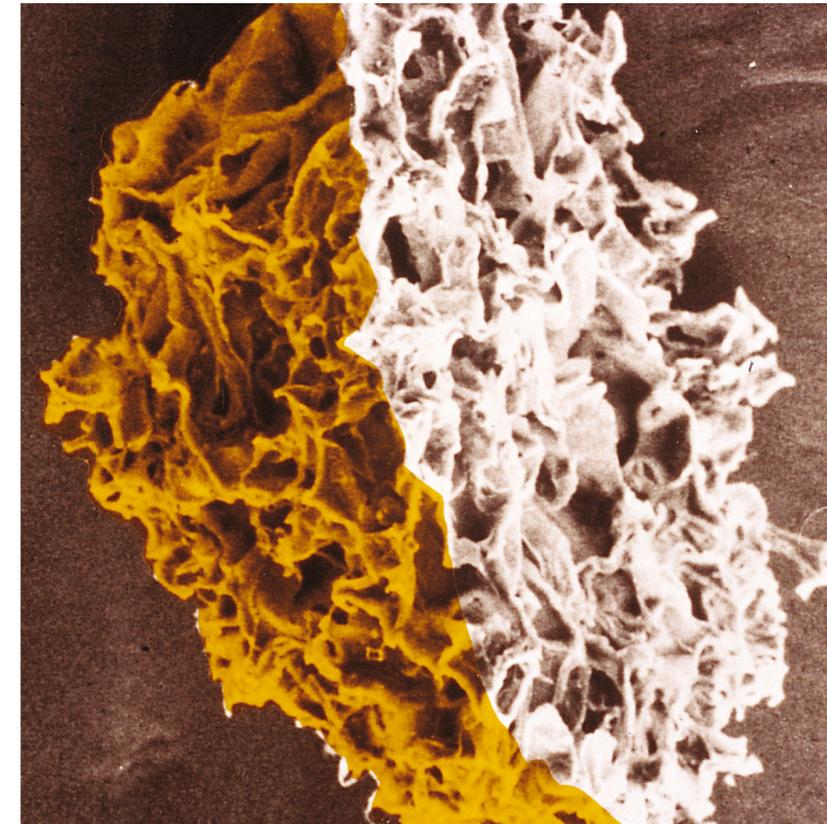
Fig 37. Cut through empty Cytopore.

Cytopore charges throughout matrix

cross-linked cellulose $-O-CH_2CH_2-N \begin{smallmatrix} CH_2CH_3 \\ \\ CH_2CH_3 \end{smallmatrix}$ .HCL
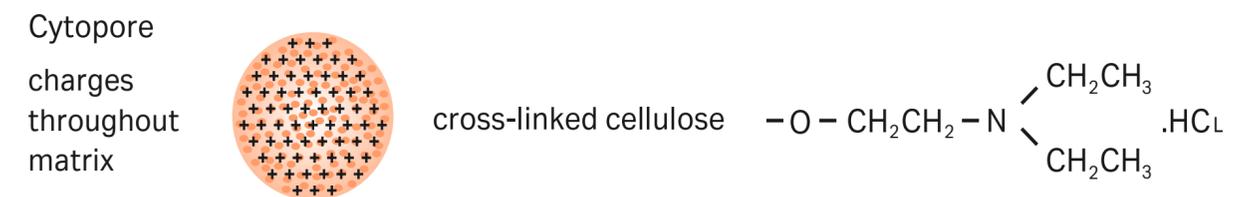
Fig 38. Schematic representation of Cytopore 1, 2 macroporous microcarrier.

Unused material can be stored dry at room temperature. Used material can be stored autoclaved and sterile for years.

The storage temperature for dry microcarrier beads is between 8° and 25°C. When stored dry between 8° and 25°C, the product will have a shelf-life of 4 years. Hydrated microcarrier beads that have been sterilized should be stored at 4°C. Hydrated microcarrier beads that have been sterilized and stored at 4°C will be stable for at least 2 years.

Properties of Cytopore 1 and 2 are listed in Table 12.

Table 12. Properties of Cytopore 1 and 2

Properties	Cytopore 1	Cytopore 2
Mean particle size	235 µm	235 µm
Particle size distribution	200–270 µm	200–270 µm
Density	1.03 g/mL	1.03 g/mL
Approx. area	1.1 m^2/g dry weight	1.1 m^2/g weight
Approx. no. of microcarriers/g dry weight	3×10^6	3×10^6
Swelling factor	40 mL/g	40 mL/g
Charged group	DEAE	DEAE
Charge density	0.9–1.2 meq/g	1.65–1.95 meq/g

The following pack sizes of Cytopore 1 and 2 are available: 20 g, 100 g, 500 g. Open and store under dry conditions. Cytopore microcarriers are stable for more than 4 years when stored unopened at room temperature. Cytopore is supplied as a dry granulated powder.

5.2.1 Cell types cultured on Cytopore

Table 13. List of cells cultured on Cytopore

Cells	Media
CHO-K1	2% FCS; 5% FCS, protein-free
r-CHO based on DHFR-	2% FCS; 5% FCS, protein-free
r-CHO based on CHO-K1	2% FCS; 5% FCS, protein-free
Murine-hybridoma	2% FCS; protein-free
Human, hybridoma SP2/0 NSO	2% FCS; protein-free
m-Fibroblasts	10% FCS
BHK-21	2% FCS; protein-free
Vero C127	5% FCS, protein-free
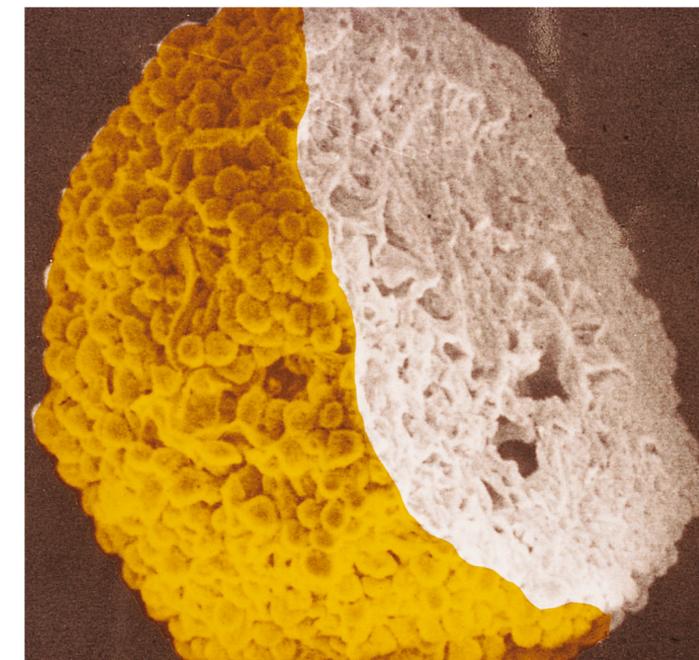
Fig 39. Cross-section through fully colonized Cytopore with CHO cells.

06
Microcarrier culture equipment

6.1 General outline of procedure

Microcarrier culture is a versatile technique for growing animal cells and can be used in a variety of ways for a wide range of applications (Section 4). Although microcarrier culture is an advanced technique, it is based on standard animal cell culture procedures and does not require complicated or sophisticated methods. Information on cell culture methods can be found in several references (156, 157, 158, 159).

Microcarrier culture procedures are based on what is already known about the cell type to be cultured. Information about morphology, plating efficiency and growth properties of a cell type in a traditional monolayer is invaluable when working out the most suitable microcarrier procedure. The best procedure is the one that ensures maximum attachment of the inoculum and results in rapid, homogeneous and high-density cell growth. The aim of Section 8 is to outline the basic principles of microcarrier culture. These principles provide the background necessary for working out the best culture procedures for a wide variety of cells without having to spend time on extensive preliminary experiments.

A general outline of microcarrier culture can be defined by several simple steps:

Step 1: Choose the most suitable microcarrier based on cell type and application (Section 4.6).

Step 2: Select the most suitable culture vessel for the application. The best results and highest yields are obtained from microcarriers maintained in suspension (Section 6), but a static culture is often used in an initial experiment (step 4).

Step 3: Hydrate and sterilize the microcarriers (Section 8.4.1).

Step 4: Conduct an initial experiment with the microcarriers in a bacteriological Petri dish. The time required for attachment of cells to the microcarriers will subsequently influence the culture procedure used during the initial stages of the culture cycle (Section 8.4.2). A rounded morphology and any tendency of the cells to clump will also define what stirring speeds to use (Section 8.4.3). A rapid fall in culture pH (the medium turns yellow within 3 days) indicates that modification of the medium may be necessary for high-density cultures (Section 8.4.3.2, 7.1).

Step 5: Carry out the microcarrier culture in the chosen vessel. The most suitable inoculation density, concentration of microcarriers and stirring speed (when applicable) can be deduced from what is known about the cell type and from the results of step 4. Section 8.4.2 and 8.4.3 describe the principles of choosing the most suitable conditions.

Step 6: Optimize the culture procedure and conditions if necessary. Occasionally, the results of step 5 indicate that further experiments are needed to increase the efficiency of the culture. The above steps are usually performed with the culture medium normally used for a given type of cell. It may be necessary to change or modify the medium (Section 8.4.3, 8.4.3.2, 7.1) and to alter the supply of gases (Section 7.2.1). In addition, greater economy of inoculum and serum may be achieved by altering the culture conditions (Section 8.4.2, 7.1.1.8). More information on optimizing culture conditions and troubleshooting is found in Sections 10 and 11.

6.2 Requirements

The best results from microcarrier cultures are obtained when using equipment that gives even suspension of the microcarriers with gentle stirring and adequate exchange of gases with the culture medium. Avoid erratic stirring as this detaches rounded mitotic cells from the microcarriers. The shape of the culture vessel and stirring mechanism should be chosen to prevent sedimentation of microcarriers in any part of the culture vessel. For this reason, vessels with slightly rounded bases are preferred.

It is important to avoid vibration so check stirring mechanisms carefully. Magnetic stirring units are often a source of vibration. However, placing a thin piece of plastic foam between the culture vessel and the surface of the base unit is a simple way of reducing vibration. Note: if magnetic stirring units are placed in humidified incubators, the electrical circuits should be isolated.

The choice of vessel for microcarrier culture depends on the purpose of the study and the desired culture volume. Laboratory-scale microcarrier cultures are generally less than 5 liters and can be contained in a wide variety of vessels. Large-scale microcarrier cultures range from approximately 5 to several thousand liters and must be maintained in specially-designed vessels (fermenters) that allow monitoring and control of parameters such as gas tension and pH.

6.2.1 Siliconizing culture vessels

Whenever glass surfaces are used with Cytodex, the inside surface of the vessel should be siliconized to prevent the microcarriers sticking to the glass. It is also useful to siliconize other glassware (e.g., pipettes, bottles, etc.) that may be used for transferring or storing hydrated microcarriers. The best siliconizing fluids are those based on dimethyldichlorosilane dissolved in an organic solvent. A small volume of siliconizing fluid is added to the clean culture vessel and is used to wet all surfaces that may come into contact with the microcarriers. Excess fluid is drained from the vessel, which is then allowed to dry. The vessel is washed thoroughly with distilled water (at least twice) and sterilized by autoclaving. One coating with siliconizing fluid is sufficient for many experiments. Examples of suitable fluids are: Sigmacote™ (Sigma Chemical Co.), Dimethyldichlorosilane (BDH) and Siliclad™ (Clay-Adams).

Siliconizing culture vessels is not needed for Cytoline microcarriers, nor is it necessary to siliconize polished stainless steel culture vessels.

6.3 Unit process system

A unit process system contains all cells used in production within the same vessel (Fig 40). This means that all cells are grown under "identical" conditions! Examples of truly homogeneous unit process systems for carriers are microcarrier cultures in stirred tanks and fluidized bed cultures with sufficient oxygen supply to not limit scalability. The Verax system is not truly homogeneous as it creates an oxygen gradient throughout the bed as the medium passes through. This makes it difficult to scale up. Other examples of unit process systems are the glass bead reactor run as a packed bed and Fibra-Cel™ woven plastic discs, also run as a packed bed. In both cases, the carriers with cells are stationary, which results in channeling of media in the reactor. Due to this channeling, necrotic zones arise inside the carrier bed.

Fig 40. Surface-to-volume ratio comparison of a unit process system (macroporous microcarriers) and a multiple unit system (rollers).

6.4 Small-scale equipment

Small-scale equipment for microcarrier cultures often comprises plastic bacterial Petri dishes for stationary cultures, roller bottles for mixed cultures, and glass spinner flasks for stirred cultures. Borosilicate glass is normally used in spinners (Fig 41).

Roller   T-flask   Shaking flask   Spinner flask
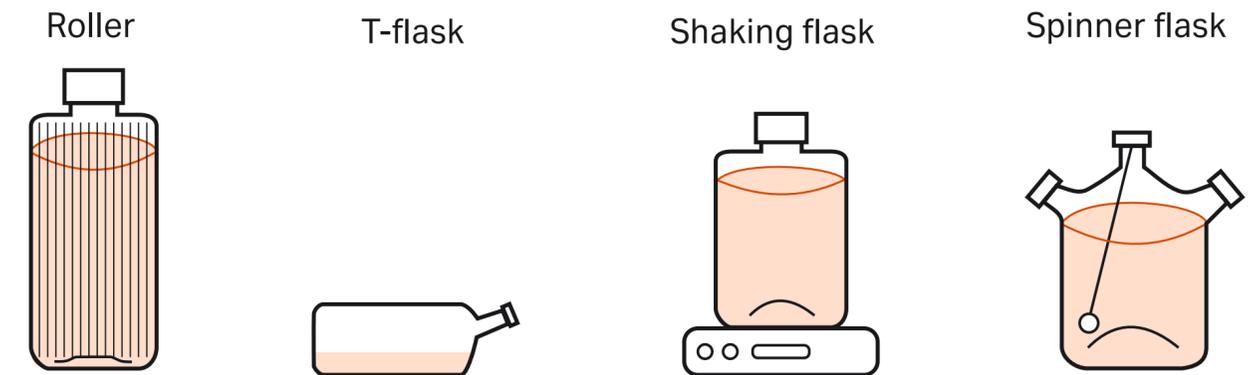
Fig 41. Small-scale equipment for microcarrier culture.

6.4.1 Spinner and rod-stirred vessels

The most suitable vessels for general-purpose laboratory-scale microcarrier culture are those with a stirring rod or impeller driven by a magnetic base unit (Fig 42). Cultures stirred by a bulb-shaped rod (Fig 42) produce higher yields of cells than cultures stirred by the spinner principle and are more suitable for cells with a low plating efficiency.

The spinner vessel has been used for many years for the suspension culture of anchorage-independent cells. The culture is stirred by a suspended Teflon-coated bar magnet driven by a magnetic stirring base unit. The stirrer bar should never come into contact with the inside surface of the vessel during culture since this may damage the microcarriers. Similarly, spinner vessels having a bearing immersed in the culture medium are not suitable, since the microcarriers can circulate through the bearing and be crushed. When using spinner vessels, adjust the position of the impeller to minimize sedimentation of microcarriers under the axis of rotation. This is usually accomplished by positioning the end of the impeller a few millimeters (approx. 5 mm) from the bottom of the spinner vessel. Spinner vessels used under closed culture conditions are suitable for cultures ranging in volume from 2 mL to 1–5 liters. If greater culture volumes are required, an open, monitored culture system is advisable (Section 6.5).

The maximum culture volume that can be conveniently used in closed spinner vessels depends on cell type, how rapidly culture conditions change throughout the culture cycle, and how often the culture medium is replaced. For example, the rapid accumulation of acid in cultures of some established cell lines requires either frequent changes of medium or other methods of controlling pH and gas tension (Section 7.3, 7.2). The capacity of the closed culture system is limited by the gas exchange possibilities (i.e., the volume of the culture, the surface area of the gas/medium interface and the volume of the gas headspace, (Fig 63, Section 8.4.3.2). While good results with many cell types can be obtained from traditional spinner vessels, more recently developed vessels and magnetic stirrers can be obtained from Bellco Glass Inc. (Vineland, NJ, USA), which has modified the traditional spinner for use with microcarriers (Fig 46). Wheaton Scientific (Milville, NJ, USA) and Wilbur Scientific Inc. (Boston, MA, USA) also supply spinner vessels and magnetic stirring base units suitable for microcarrier culture (69). Culture vessels modified with rounded bases can be supplied on request (Fig 42).

(A) Traditional spinner vessel of type supplied by Bellco Glass Inc. or Wheaton Scientific. Stirring speed 50–60 rpm.

(B) Spinner vessel modified for microcarrier culture: paddle impeller (Bellco Glass Inc.). Stirring speed 20–40 rpm.

(C) Rod-stirred microcarrier culture contained in a vessel with indented base (Techne (Cambridge) Ltd.). A magnet is present in the bulb of the stirring rod. Stirring speed 20–30 rpm.

(D) Spinner vessel modified for microcarrier culture: plough impeller. Vessels with round bases were supplied by Wheaton Scientific and the plough-shaped impeller was fashioned from PTFE. Stirring speed 15–30 rpm. (Hirtenstein, M., Clark, J.M., Gebb, Ch., Adv. Exp. Med. **172**, 151–167 (1984), by kind permission of the authors and the publisher.)

Fig 42. Various stirred culture systems for laboratory-scale microcarrier culture. All systems are stirred by a magnetic base stirring unit.

An inexpensive spinner vessel for culture volumes between 2 and 20 mL can be made from scintillation vials (160). Such a vessel can be used with magnetic stirring base units where the stirring speed ranges from approximately 10 rpm to 40 rpm. An improved principle for keeping microcarriers in suspension is the asymmetric stirring action provided by a suspended stirring rod. Techne (Cambridge) Ltd. (Duxford, Cambridge, UK) has developed a technique for stirring microcarrier cultures where a bulb-shaped rod with one end fixed above the culture moves with a circular motion in a culture vessel with a rounded and indented base (Fig 42). This system provides a more gentle and even circulation of microcarriers and eliminates the sedimentation of microcarriers often observed when using spinner vessels (161). The rod-stirred microcarrier vessel is used in combination with a low-speed magnetic base stirring unit and results in yields of cells significantly greater than those achieved with spinner vessels. This increase is particularly apparent for cells with low plating efficiency (Fig 42). Such a system can be used for cultures with volumes ranging from approximately 100 mL to 3 liters.

The spinners with the best performance for microcarriers are the Techne bottles equipped with bulb stirrers. The spinner flasks are placed on magnetic stirrers in incubators or warm rooms. Normally, there are no controls attached. However, the Superspinner supplied by B. Braun does incorporate silicone tubing attached to a pump to increase oxygen supply (Fig 43). Spinners are available up to 20 liters, but it is easier to handle 4 × 5 liter flasks in incubators and sterile workbenches. This volume is sufficient to generate inoculate for 1–200 liter cultures.

Fig 43. B. Braun Superspinner.

6.4.2 Roller bottles

Although spinner and rod-stirred cultures provide maximum yields from microcarriers, microcarriers can also be used to increase the yield of cells, virus or interferon from roller bottles. Under roller bottle conditions, the yield of cells per unit weight or unit area of Cytodex is approximately 50% of that under optimal conditions in rod-stirred cultures. Nevertheless, by using Cytodex microcarriers, it is possible to obtain a 5- to 10-fold increase in growth area in each roller bottle. This increase in growth area is paralleled by a corresponding increase in cell number and yield of virus or interferon. The yield depends on the availability of nutrients from the medium, control of pH and an adequate supply of oxygen (see below). There are three methods for using microcarriers in roller bottles.

Method 1: Cytodex used to increase the yield from roller bottles using standard roller techniques

The microcarriers adhere to the surface of the roller bottle and provide a fixed surface for cell growth. The amount of microcarrier added is not critical, but approximately 1.5 mg/cm^2 of roller bottle surface is adequate. Microcarriers can be added with the initial supply of culture medium. It is not necessary to remove unbound microcarriers, and the rolling speed should be that normally used for a given type of cell. This method can only be applied with Cytodex and Cytopore.

Method 2: Microcarriers used for "suspension" culture in roller bottles

Siliconized roller bottles (Section 6.2.1) should be filled 1/2 to 2/3 full with medium containing Cytodex at a concentration of not more than 3 mg/mL (Cytopore 2 mg/mL, Cytoline max 50 mL per 500 mL medium). The bottles are inoculated with the usual number of cells used for suspension microcarrier cultures (Section 8.4.2.4), flushed with a mixture of 95% air and 5% CO$_2$ and sealed. The most suitable speed of rotation is greater than that normally associated with roller bottle culture. A speed of 5–15 rpm should be sufficient to ensure good but gentle stirring. Stringent aseptic techniques are required since the medium comes into contact with the inside of the bottleneck and cap. Modified culture procedures (Section 8.4.2.2) can be used with this method.

Method 3: Cells grown to confluence in rotating roller bottles using standard techniques

This method was developed by L. Kronenberg (103, patent pending). Once confluence is achieved, the culture medium is replaced by fresh medium containing microcarriers. Approximately 1.5 mg Cytodex in 0.5 mL medium/cm^2 of roller bottle surface is adequate. The bottles are rotated at 2–4 rpm for approximately 15–30 min, during which time an even layer of microcarriers adheres to the confluent layer of cells. The rotation speed is then reduced to that normally used for roller bottle culture of the given type of cell (0.25–1 rpm). Cells migrate onto the microcarriers and form additional confluent layers while the original monolayer on the surface of the bottle remains stable. This technique effectively accomplishes two subcultures in the one vessel and allows a 50–75% reduction of medium consumption per cm^2 or per 10^6 cells (103). Replenishing the medium is not usually necessary during the microcarrier phase of the culture.

The choice of method depends on the circumstances and cell type. Method 2 requires rolling machinery capable of higher speeds of rotation. With this method, yields for certain types of cells may be limited by the ability to control pH in the closed roller bottle.

The success of Method 3 depends on the ability of cells to migrate from the confluent monolayer onto the microcarriers. It is, however, the procedure of choice since the technique is simpler and more reliable than the other two. The microcarriers are also fixed or immobilized (Fig 44), which means that such cultures are compatible with common virus/interferon harvesting schemes used with roller bottles. Method 3 has been used to increase the yield of interferon from roller bottle culture (103) Note, however, that more media changes and oxygen are needed due to the higher cell densities.
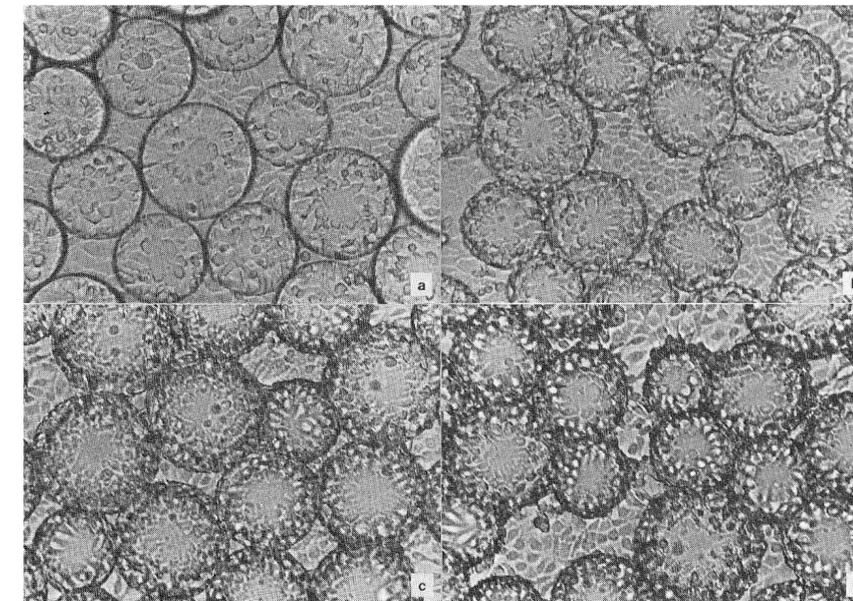
Fig 44. Roller bottle cultures of mouse L-cells using Cytodex to increase the yield. Confluent cultures were drained and replenished with 400 mL medium containing 1.5 g Cytodex 1 (roller bottles approx. 1400 cm^2). The monolayers were photographed at a) 8 h, b) 24 h, c) 72 h and d) 96 h. (Original photographs by L. Kronenberg, Lee Bio Molecular Res. Labs. Inc., San Diego, USA, reproduced by kind permission.)

6.4.3 Rocking bottles shaker flasks

The yield of cells from culture bottles (e.g., Roux bottles) can be increased several fold by using Cytodex, Cytopore or Cytoline. The microcarriers can be easily kept in suspension by using a rocking platform to keep the culture in motion, and by supplementing the medium with 5–10% (w/v) Ficoll™ 400 (S. Smit, pers. comm., 162). Ficoll 400 increases the density of the culture medium and thus allows the microcarriers to float more easily. The procedures outlined in Section 8.4.2 apply to this culture method. The rate of rocking should be just sufficient to ensure slow movement of the microcarriers. Make sure that the bottles you are using have enough head space for oxygen transfer. Figure 45 demonstrates the best combination (roller bottle 450 cm^2 on an orbital shaker), especially if testing Cytopore.
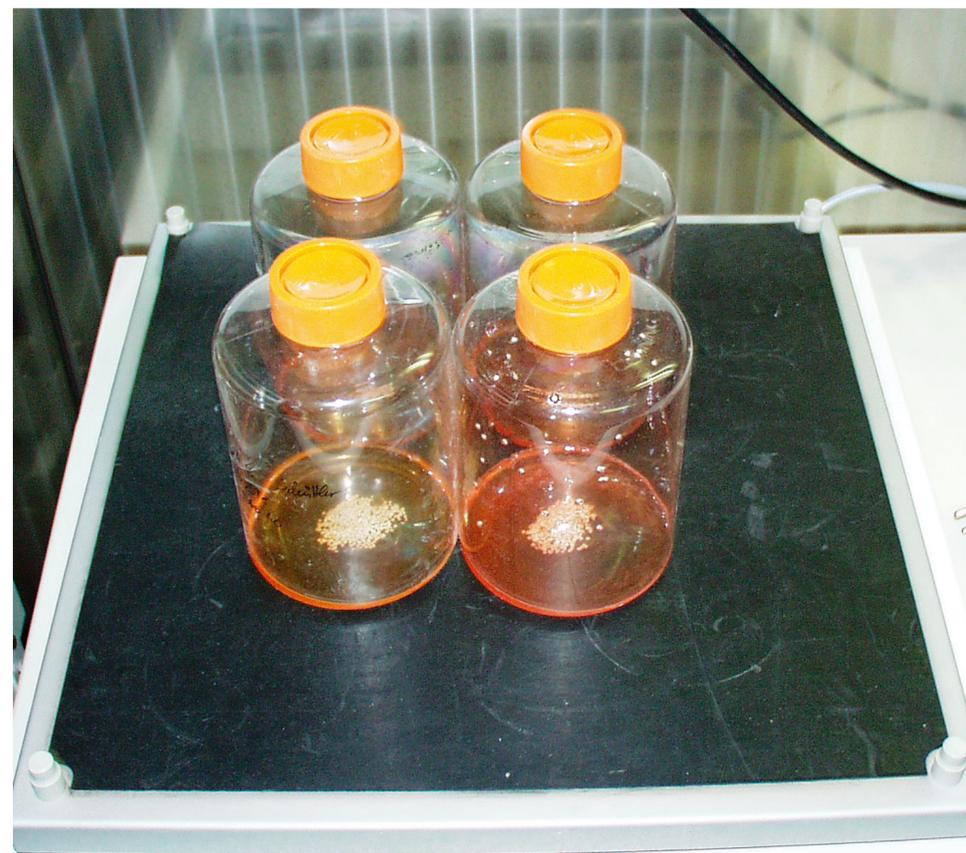
Fig 45. Small-scale macroporous microcarrier test configuration (a). Large bottles (e.g., 450 cm^2 roller bottles) on an orbital shaker (b).

6.4.4 Air-lift and fluid-lift culture systems

An alternative method for keeping microcarriers in suspension is to use an upward flow of air or culture medium through the culture. The success of air-lift microcarrier culture depends on using gas of high purity and on defining the gas requirements of each cell type (Section 7.2). Only those cell types that remain relatively strongly attached to culture surfaces during mitosis can be grown with this method. The erratic movement caused by the gas bubbles causes greater shear-forces than when using circulating culture medium to achieve suspension. A simple fluid-lift microcarrier culture system is illustrated in Figure 46. After inoculation of cells into such a system, a static culture period is necessary. Once the cells have attached, circulating medium can be used to keep the microcarriers in suspension. This system produces somewhat better cell yields than spinner vessels (78), but is more difficult to use for general culture under aseptic conditions.

6.4.5 Small-scale perfusion culture

Cytodex or Cytopore can be used to greatly increase the culture surface area of perfusion chambers. Alternatively, an efficient perfusion culture can be constructed by filling a chromatographic column with microcarriers covered with cells and then perfusing medium through the column. Such a system is illustrated in Figure 25.

6.4.6 Dishes, tubes and multi-well trays

The surface area of cell culture Petri dishes and wells can be greatly increased by using microcarriers. Adding Cytodex (5 mg/mL) improves the yield from Petri dishes by at least two-fold and the yield from wells by five-fold. Microcarriers can be added as a suspension in the culture medium and the dish or well inoculated with the usual number of cells. With this procedure, the microcarriers do not attach firmly to the surface of the culture vessel and the culture should be aspirated gently with a pipette every few days to avoid extensive aggregation of microcarriers. Alternatively, microcarriers can be added in PBS to the cell culture dish or well. They attach to the culture surface after a few minutes, after which the PBS is removed and the dish or well rinsed carefully with culture medium. The culture is then inoculated in the usual manner.

Cytodex can also be added to confluent dishes for subculturing without enzymes or chelating agents (Section 4.5.2). With this method, cells migrate from the confluent monolayer onto the microcarriers, which can then be removed to inoculate other cultures or for biochemical studies.

Bacteriological Petri dishes are useful when the growth of cells is restricted only to the microcarriers. Such a culture is a valuable first step when working for the first time with the microcarrier culture of a particular type of cell (Section 8.1). For such a preliminary experiment, the dish should contain 3–5 mg Cytodex/mL and be inoculated with approximately $1-2 \times 10^5$ cells/mL. Culture tubes can also be used. However, to ensure even growth of cells on the microcarriers, the tubes must be kept in smooth motion. Nilsson and Mosbach (81) successfully used an end-over-end rotator for growing a variety of cells in tube microcarrier cultures.
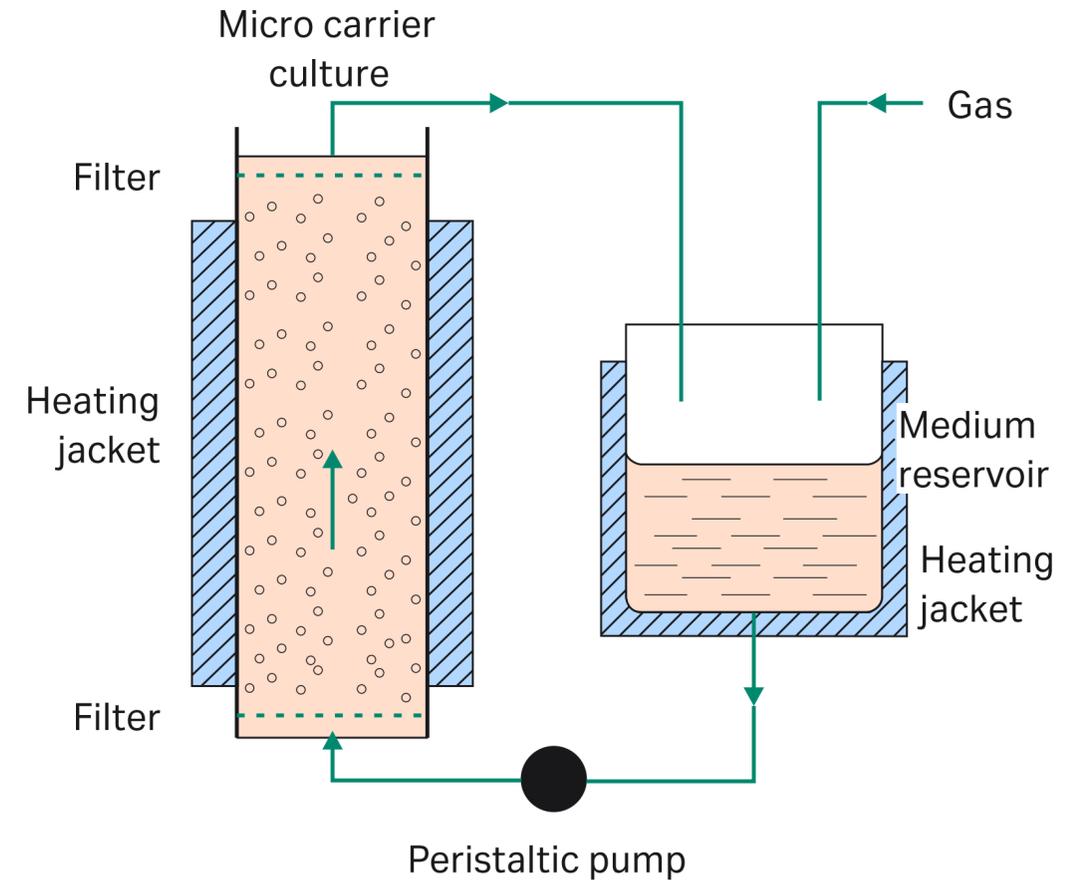
Fig 46. Schematic diagram of fluid-lift microcarrier culture system. The filters should have a mesh sufficient to exclude Cytodex microcarriers (approx. 100 μm). An example of this type of system is described in reference 78. The culture can be contained in a chromatographic column with medium circulated at 17 mL/min.

6.4.7 Bench-scale fermenters

For process development, a number of 1- to 10-liter fermenters (at least two) are used (Fig 47), usually made of borosilicate glass. To prevent cells and positively charged carriers attaching, the glass needs to be siliconized and the silicone solvent washed away before use.

The temperature of the fermenter is controlled by circulating water between the double jacket and a waterbath, or by a heating jacket. There is also complete pH, dO_2, and stirrer control. Often, the controllers are also equipped with pumps for adding alkali and/or medium perfusion. The fermenter bottom should be round to give better mixing, and a marine impeller used to minimize shear forces. A spin filter is needed to run perfusion and sparge gas into the culture. In smaller reactors, this can be mounted on the stirrer axis. The mesh size is normally between 60 and 120 μm.

Fig 47. B. Braun laboratory fermenter.

6.5 Large-scale equipment (culture vessels)

6.5.1 Large-scale stirred tank microcarrier culture vessels

The requirements for large-scale microcarrier culture equipment are similar to those for small-scale, but because of the large culture volumes involved, equipment for monitoring and controlling several culture parameters is required. A variety of different configurations have been used successfully at large scale (1, 4, 7, 70, 71, 79, 80, 97, 98, 163). However, to date the most suitable commercially available system has been a modified "Bilthoven Unit" (164) supplied by Contact Holland (Ridderkerk, Holland). Such units have been used for culture volumes up to several hundred liters (79, 80) for production of cell products such as interferon and virus vaccines.

Large-scale equipment for culturing suspension cells must be modified to account for the suspension properties of the microcarriers, slow stirring speeds and the culture procedures discussed in Section 8.4.2. Optimal function of the large-scale process usually requires that specific design features are adopted for each application and situation.
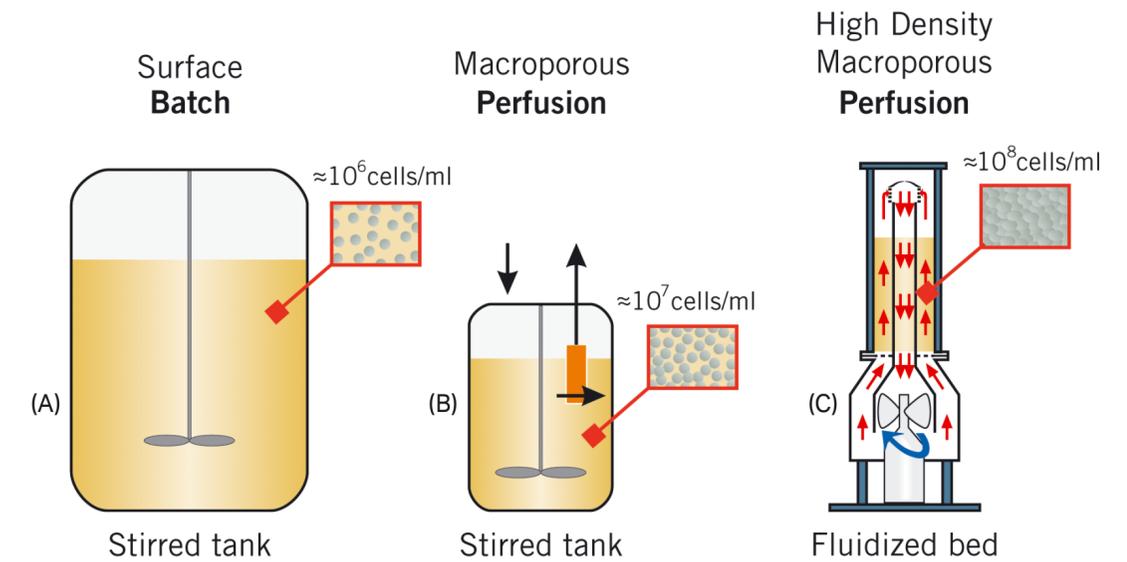
Fig 48. Different large-scale configurations for microcarriers. (A) Stirred tank batch — Cytodex. (B) Stirred tank perfusion — Cytopore. (C) Fluidized bed — Cytoline.

Larger stirred tanks are normally made of electropolished pharmaceutical grade stainless steel. Before using a stainless steel vessel for the first time, wash it with a mixture of 10% nitric acid, 3.5% hydrofluoric acid and 86.5% water. Both specialized sampling devices and spin filters are commercially available for larger reactors. A conical-shaped reactor offers a number of benefits (Fig 49). As the probes are placed low in the reactor, it is possible to vary the volume in the reactor over a greater range and still retain control. In addition, there is a large headspace to surface area ratio for gassing.

The large stirred tank reactor is the most widely used reactor in the fermentation industry and has already been scaled up to 15 000 liters. Its major advantages are its conventional design, proven performance, homogeneous results, and good potential for volumetric scale up. Disadvantages are that it is a low intensity system with high shear forces at large scale and oxygen supply limitations.

6.5.2 Packed beds

There are two different approaches to this technology. The first is to pack a cage with macroporous microcarriers (Cytopore, Cytoline) and then place it inside a stirred tank. Medium is circulated through the cage using a marine impeller. Gas is supplied via sparging. Reactors of this type are available from New Brunswick (USA) and Meredos (Germany). The second approach is to pack Cytopore or Cytoline into a column and pump medium through the column. Oxygenation is done outside in the medium loop.

Low surface shear, high unit cell density and productivity, and no particle/particle abrasion are some of the major advantages of packed beds. However, poor oxygen transfer, channel blockage and difficulties in recovering biomass from the bed have all limited their use.

Fig 49. Example of a conically shaped stirred tank reactor.

6.5.3 Fluidized beds

The external circulation system and Cytopilot are examples of two different designs of fluidized beds.

6.5.3.1 Fluidized bed with external circulation

Verax introduced this technology for animal cells (165). The fluidized bed is equipped with an external recirculation loop connected with a gas exchanger (hollow fiber cartridge), heating elements, pO_2, pH and temperature sensors, and a recirculation pump. Certain aspects of the external circulation loop can be problematic, such as shear stress created by the pump, oxygenator fouling, and sterilization procedures in large-scale. A gas exchanger transfers oxygen to the culture medium in the external loop. A certain oxygen tension at the entrance of the bed is achieved. The supernatant is increasingly depleted of oxygen on its way up the fluidized bed from the bottom of the reactor to the top (Fig 50). The oxygen tension requirement of the biological system thus limits reactor height and scalability. Other groups are working with their own designs of fluidized beds (166, 167).

6.5.3.2 Cytopilot – fluidized bed with internal circulation

A fluidized bed with internal circulation, Cytopilot was developed at the Institute for Applied Microbiology in Vienna, Austria, in co-operation with the company Vogelbusch GmbH, Vienna, Austria (168). Cytopilot comprises a lower and an upper cylindrical chamber (Fig 51). The lower chamber has a bottom adapted to the special flow conditions and is equipped with the following: a double-jacket heating circuit, sampling and discharge facilities, a magnetic stirrer (rotating in both directions), and probe nozzles for pH and dO_2. The liquid agitated by the magnetic stirrer is conveyed via the distributor plate to the microcarriers in the upper chamber of the vessel. The hydrodynamic pressure lifts the settled microcarriers to form a fluidized bed with a clear boundary between the top of the fluidized microcarriers and the uppermost part of the medium volume. The bed expands or contracts as a function of the stirrer speed. The medium then flows through a sieve to the internal recirculation loop and back to the stirrer in the lower chamber of the vessel. Micro-bubbles of oxygen are sparged homogeneously into the downflow in the draft tube and then uniformly distributed by the impeller (Fig 51). This system provides oxygen gas hold-up via the dispersed oxygen bubbles (continuous transfer of dO_2 from gas to liquid). It both minimizes dO_2 gradients in the system and greatly increases the theoretical height of the fluidized bed.
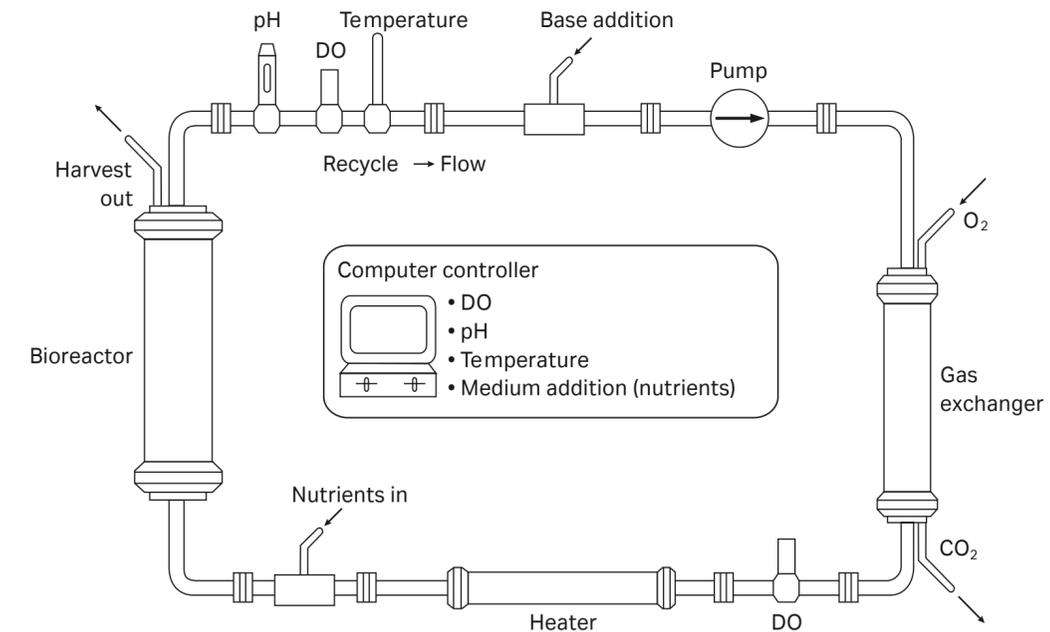
Fig 50. Fluidized bed with external re-circulation.
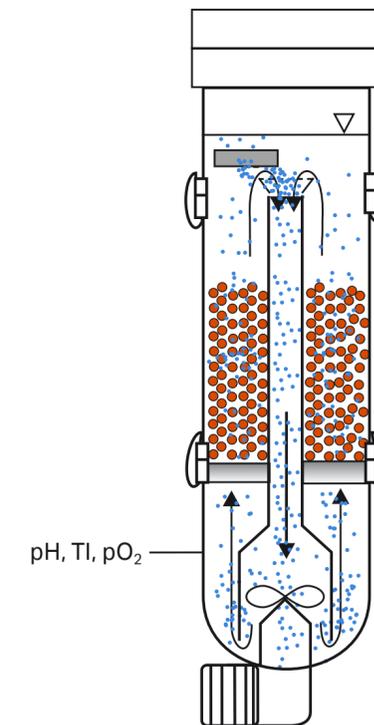
Fig 51. Fluidized bed with internal circulation.

6.5.3.3 Fluidization and fluidization velocity

A carrier bed lying on a distributor plate through which medium flows will have a pressure drop (Δp). This pressure drop reduces the pressure (p) of the carrier bed on the plate. If Δp equals p, the bed will expand (Fig 52). This is the fluidization point. The maximum fluidization speed depends on the sedimentation rate of the microcarriers. When the upward flow rate is higher than the sedimentation rate, microcarriers will be carried to the top. This is known as flush-out.

Flush-out velocities are between 10 and 100-fold higher than the velocity at the fluidization point. The efficiency of mixing in a fluidized bed is much higher than in many other systems. This efficiency increases with the particle size of the carriers.

Because there are no channels between carriers for cells to block, and because it allows cells to be retrieved from the reactor, the fluidized bed represents an improvement over the packed bed reactor. Scale-up potential and very good mass transfer are additional benefits. Drawbacks include particle/particle abrasion and shear stress affecting growth on the bead surface (This affects only 10% of the available area of macroporous beads.)
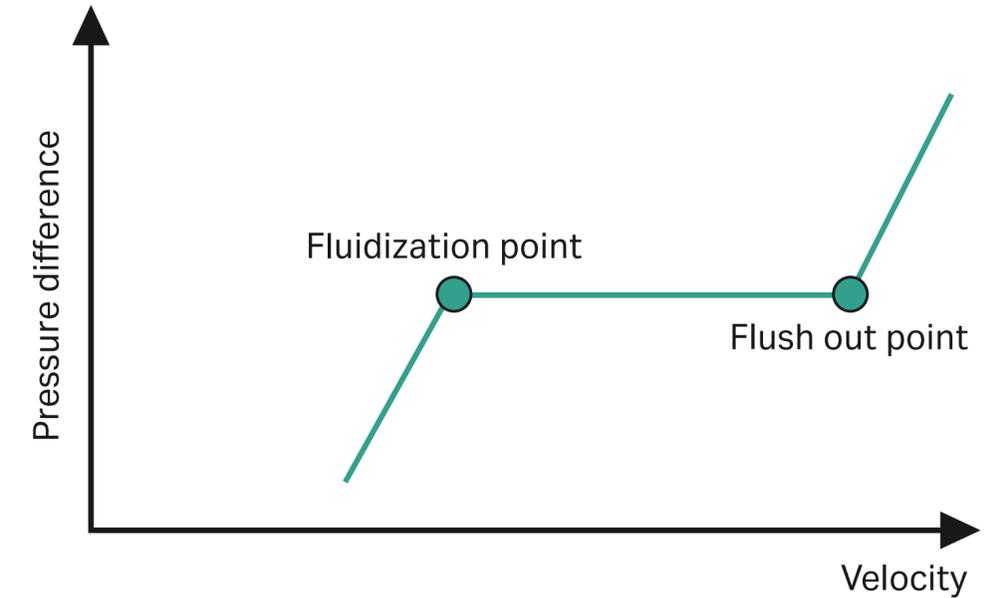
Fig 52. Fluidization principles.

6.6 Retention systems

To overcome the production limitation of continuous cultivation, organisms must somehow be retained in the reactor. Such a process would increase cell density and dilution rate (perfusion rate is the correct expression), both of which increase productivity. A retention process would uncouple the supply of nutrients and the withdrawal of inhibiting products from the growth rate of the cell line in the reactor. This is the principle of a so-called perfusion reactor. Not only is the perfusion rate independent of the maximum cell doubling time but also the product is separated from the producing cells, which could be very important with unstable products.

Most cell retention systems can be used to retain microcarriers. Problems occurring with cells in suspension in some retention systems, e.g., filter fouling and nutrient limitations due to long settling times, are reduced using microcarriers. Cells are attached to the beads and therefore the settling time is much faster. In addition, the mesh sizes of the retention filters can be much bigger.

6.6.1 Spin filters

The spin filter (also called rotating sieve or rotor filter) represents another way to avoid membrane fouling. When Taylor vortices form in a narrow cylinder, they create the necessary turbulence in the vicinity of the sieve to prevent solids from settling. Centrifugal forces might also have a positive influence. The spin filter is very popular in animal cell cultivation and is found in many production processes and design variations. The membrane consists of a cylindrical stainless steel sieve available with different mesh sizes. The permeate is not completely cell free.

The mesh size should be according to the size of the particle you want to separate. For microcarriers, especially Cytodex and Cytopore, a mesh size of 60 to 100 µm is sufficient. Mesh size depends on rotation speed. The faster it rotates, the larger the mesh size. Note: increasing rotation speed also increases shear stress. When placing the spin filter within the reactor, the system does not require a pump for recirculation of broth. The by-pass system can be separated, cleaned, sterilized and reconnected to the reactor. Twin filter systems enable switch-over to a second spin filter while the first is cleaned. Magnetic drive gear pumps, diaphragm pumps and peristaltic pumps circulate the broth without damaging cells (Fig 53).

6.6.2 Settlers

Settlers are non-moving devices that separate phases using different sedimentation rates. They make it easier to separate microcarriers than cells because the sedimentation rate increases with microcarriers. Settlers can be used inside and outside the reactors. The simplest settler for stirred tank reactors is just a tube with a connection for the harvest line. This breaks turbulence inside the reactor so that you can draw off the medium. These devices are suitable for Cytoline, which has a high sedimentation rate, and for Cytodex and Cytopore if the cell density is not too high. If the perfusion rate exceeds a certain level, use a settler with the configuration shown in Figure 54. This settling device can be scaled up by increasing the diameter in relation to the size of the reactor.

Fig 53. External spin filter. Courtesy of Bioengineering AG, Switzerland.

L = Lamellas
C = Cell suspension from reactor
S = Split flow to clarification zone
V = Settled viable cells
P = By-pass containing settled viable cells
H = Harvest medium containing dead cells
R = Recycle to reactor
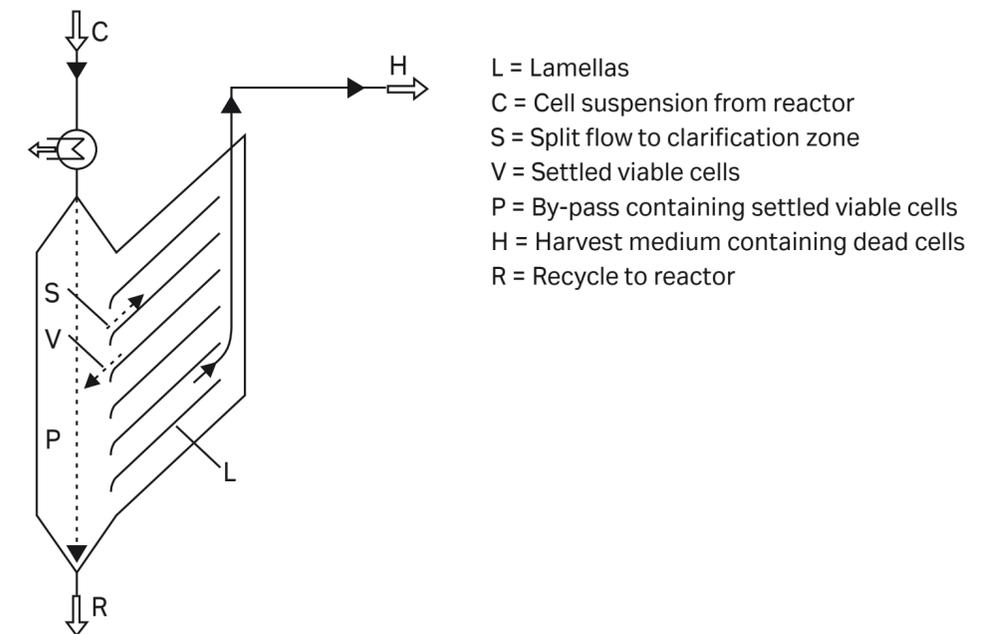
Fig 54. Example of a settler design. Lamellar clarifier for perfusion culture systems. J. Stereny, Univ. Dortmund, Germany.

6.6.3 Ultrasonic device

Ultrasonic retention can be used as a microcarrier retention system for Cytodex and Cytopore (Fig 55). The ultrasonic cell separation chamber (BioSep ADI 1015 (Applicon, The Netherlands) (169) operates like a cell retention filter (170). Bernoulli force and the secondary radiation force bring the microcarriers closer together in the retention chamber. Aggregates develop and, as a result, the Stokes drag increases and sedimentation finally forces the microcarriers back to the bioreactor where they are re-suspended by stirring. This system was primarily developed for cells but it is tested for Cytodex (pers. comm. Felix Trampler) as well. The device's main problem is scalability.

6.6.4 Hollow fibers

Hollow fiber membranes are applied in the medical field, for hemodialysis of patients with kidney failures, for instance, and in laboratory and industrial processes such as the separation and purification of gases, liquids and solids. More recently, hollow-fiber reactors have been used for compartmentalizing enzymes and culturing microbial, plant and animal cells. Membranes and reactors with specific properties have been developed for these applications. The filters used for cell retention are suitable for microcarrier retention as well. For this purpose, external hollow fibers are used in a re-circulation loop.

Stirred tank culture with Cytopore as example:

CHO cells grown on Cytopore 1 microcarriers and a 0.2 µm, 110 cm² Hollow Fiber Cartridge (Cytiva CFP-2-F-3MA) was used for carrier retention. The reactor working volume was 5.5 L, perfusion rate was 1–3 L per day. The cartridge was integrated in an external loop. A peristaltic pump was used for the flow of the cell suspension and permeate flow was controlled by a second peristaltic pump. Crossflow rates from 100 to 250 mL/min is useful to achieve permeate flow rates of up to 10 mL/min. Cytiva membranes are used for the ATF technology for perfusion (Refine Technology) (Fig 56).
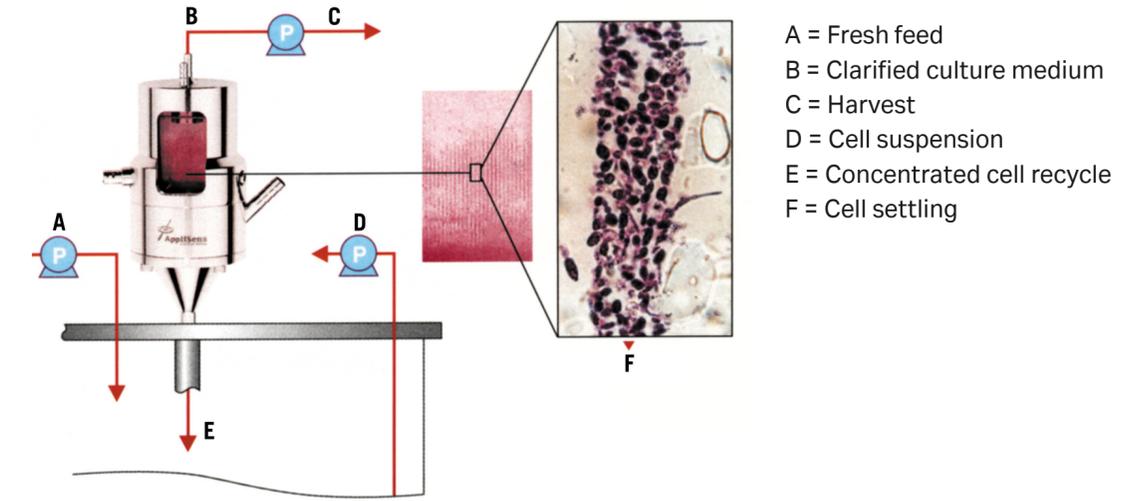
A = Fresh feed
B = Clarified culture medium
C = Harvest
D = Cell suspension
E = Concentrated cell recycle
F = Cell settling

Fig 55. Typical configuration of acoustic cell retention system from Applikon.
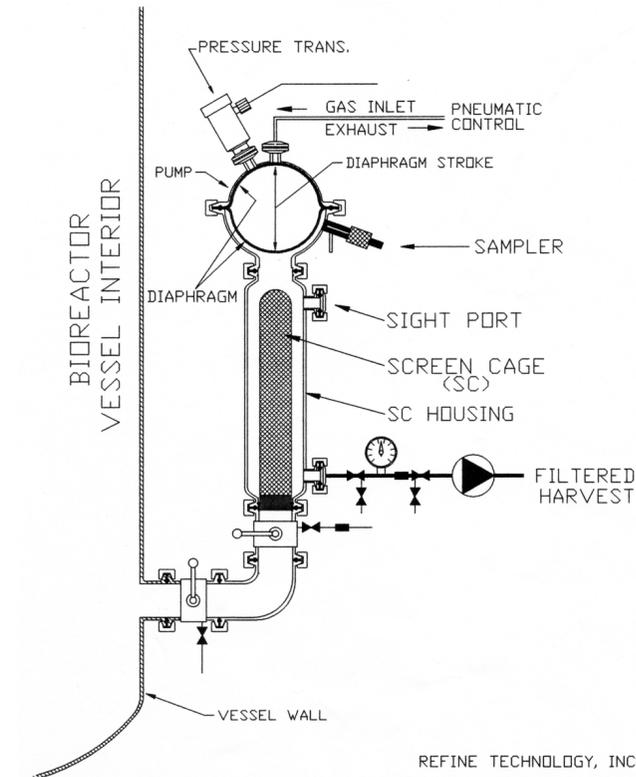
Fig 56. ATF technology for microcarrier retention.

6.6.5 Centrifuges

Centrifugation separates materials of different density when a force greater than gravity is applied (Fig 57). In bioprocessing, centrifugation is used to remove cells and cell debris from fermentation broth. Centrifugation is most effective when the particles to be separated are large, the liquid viscosity low and the density difference between particle and fluid great. That is exactly the case when using cells immobilized on microcarriers. Steam sterilizable centrifuges are applied when either the cells or fermentation liquid is recycled to the fermenter. Current centrifuges can be found inside and outside of the fermenter. Disadvantages of centrifugation include equipment cost and complicated handling, especially at large scale.

Fig 57. Sorvall centritech lab-scale perfusion module.

07
Culture conditions

7.1 Media and components

7.1.1 Choice of culture medium

A wide variety of different media can be used for the culture of any given type of cell. If the culture medium supports the growth of a particular type of cell in other systems, it will usually support growth on Cytodex microcarriers. Therefore, when selecting a medium for microcarrier culture, the most suitable starting point is the medium previously reported to support the growth of that cell type. Once the basic procedures for growing a cell type in microcarrier culture have been resolved, the medium may need to be modified if maximum yields are to be obtained. Such modifications may be necessary simply because the microcarrier culture cycle usually spans a wide range of culture densities and the supply of nutrients must take into account the different requirements for growth at different culture densities.

A richer medium is often needed for the initial stages of a microcarrier culture, especially if low cell densities are used. In such cases, the cells must survive under almost cloning conditions with only a few cells/cm^2. An additional adverse effect is stirring because it eliminates the formation of "micro-environments". Micro-environment means that cells growing close together are able to exchange autocrine growth factors, for example in static cultures or in pores of macroporous microcarriers. Since the cells have very little conditioning effect on the medium at this stage, one way to improve growth is to use a medium that contains components important for growth at low density. These are particularly important for cells with low plating efficiencies. The culture medium must have a large reserve of essential nutrients to support cell growth towards the end of the culture cycle when more than 10^6 cell/mL are usually present (Section 8.4.3.2).

Figure 58 shows that different media support the growth of cells in microcarrier culture to different extents. When cultures are initiated at a low density, Medium 199 often results in higher plating efficiencies and gives better yields than either DME or BME. In contrast, DME supports growth to much higher densities than BME or Medium 199, presumably because the concentrations of certain essential amino acids and vitamins are several-fold greater in DME than in the other media (155).

Medium composition is influenced by the tissue origin of the cells. Usually, a medium based on DME is suitable for the microcarrier culture of most types of cells. Section 5.1.3 lists the types of cells cultured on Cytodex microcarriers, a major proportion of which were cultured using DME as the base medium. To improve the growth of cells during the early stages of the culture cycle, the DME can be supplemented with components that improve plating efficiency and growth at low densities. The medium composition recommended for different cell cultures can be found in the literature and web pages of media suppliers.

When culturing cells with low or intermediate plating efficiency (i.e., less than approx. 30%), the basic DME medium is supplemented in the initial stages of culture until the medium is replenished. Non-essential amino acids are the single most important supplement for improving plating efficiency and growth in low-density cultures (Fig 59). Modifications to the initial culture procedure will also improve yield (Section 8.4.2.2). Microcarrier cultures are usually replenished with the
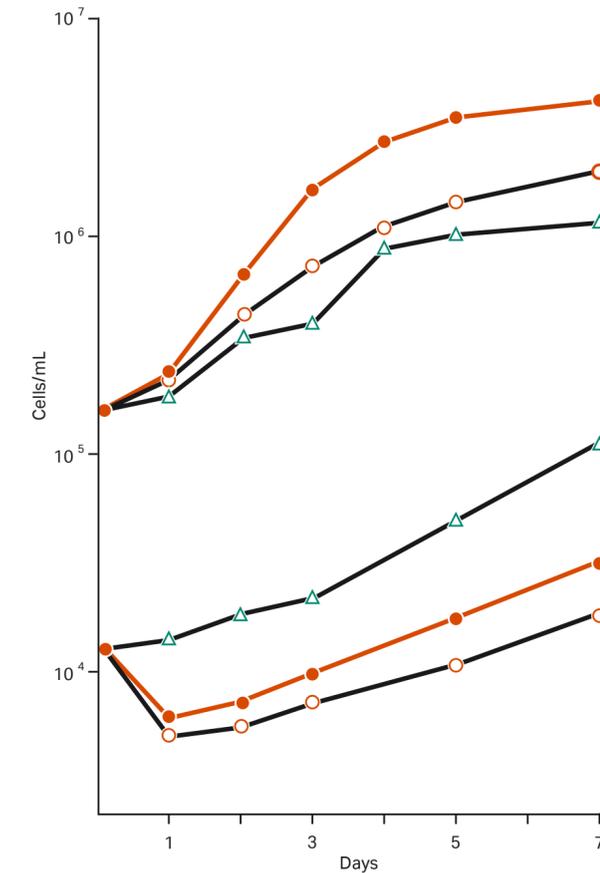
Fig 58. The effect of various culture media on the growth of monkey kidney cells (Vero) on Cytodex microcarriers. (DME, ─●─; BME, ─○─; Medium 199, ─△─). All media were supplemented with 10% (v/v) fetal calf serum. Cultures contained 5 mg Cytodex 1/mL and were stirred at 60 rpm for the culture period. In all experiments 50% of the medium was changed on day 3. (Clark, J.M., Hirtenstein, M.D., *Annals N.Y. Acad. Sci.* **369**, 33 (1981) by kind permission of the authors and publisher.)
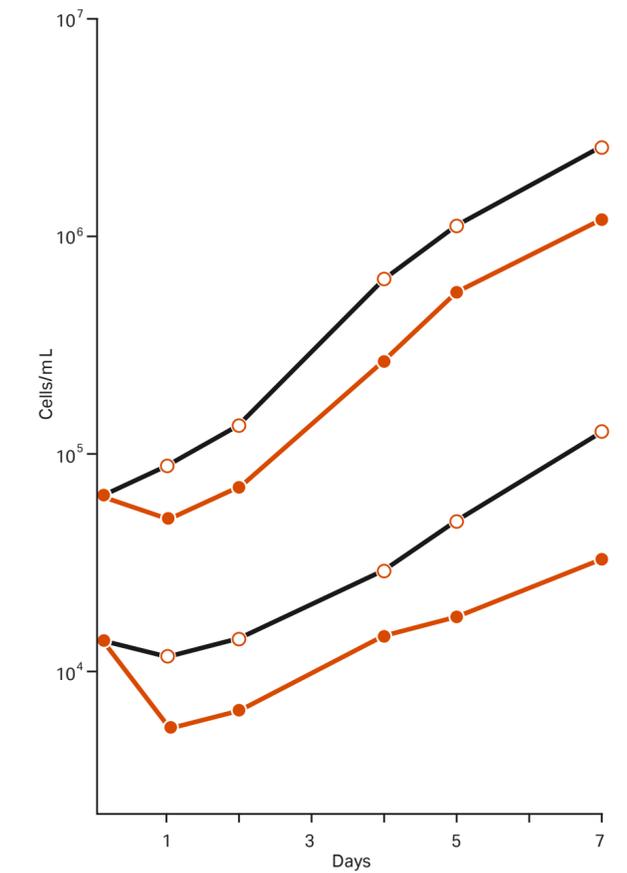
Fig 59. The effect of nonessential amino acids on the growth of monkey kidney cells (Vero) on Cytodex microcarriers. The culture media were DME supplemented with 10% (v/v) fetal calf serum (─●─) or DME supplemented with 10% (v/v) fetal calf serum, alanine, asparagine, aspartic, glutamic acid and proline; all 10^{-4}M (─○─). Cultures contained 3 mg Cytodex 1/mL and were stirred at 60 rpm for the entire culture period. In all experiments 50% of the culture medium was changed on day 3. All cultures contained 20 mM HEPES. (From Pharmacia Biotech AB, Uppsala, Sweden.)

basic formulation of DME. Under conditions when very high cell densities are being cultured (more than approximately 3×10^6 cells/mL), or when the cultures are rapidly dividing during the later stages of the cycle, e.g., many established cell lines, the replenishment medium is supplemented with additional inositol and choline. This increased concentration is important if the replenishment medium contains only low concentrations of serum (Section 8.4.3.2). Increasing the concentration of these two components can reduce the frequency or extent of medium replenishment, provided other factors do not become limiting (Section 8.4.3.2 and 7.3). Buffer systems for the control of pH are discussed in Section 7.3.2.

7.1.1.1 General comments on components of culture media

While the requirements for cell growth in microcarrier culture are similar to those for other monolayer methods, several media components need to be considered if culture conditions are to be optimized.

It is important to note that a certain amount of medium will only generate a finite amount of cells of a particular line. This is independent of the culture technology chosen! Basic media formulations were originally DME and DMEM with added F10/12 or medium 199 enrichments. RPMI 1640 was often the basis for hybridomas.

Today, there is a multitude of different media developed for specific cell types, many of them serum-free. A number of suppliers provide collapsible plastic bags with ready-made media to be hooked up to the bioreactor. The most recent development is liquid concentrates that can be automatically mixed with water on-line, sterilized and fed into the bioreactor. This system is available from Gibco.

Media composition during growth and during maintenance of the culture can be quite different. Serum is often reduced or even completely removed at later stages of high cell density cultures. For some cell lines, it is even possible to run protein-free media at later stages of the culture.

7.1.1.2 Practical aspects of culture media

The water and reagents used to prepare culture media should be of the highest possible purity (WFI). All glass bottles used for medium storage should be high-quality glass with a low content of heavy metals, and they should be well washed (171). To improve reproducibility of microcarrier culture, all procedures for preparing and supplementing culture media should be standardized, and serum supplements should be added as a stock solution just prior to culture. Additional information on use and storage can be obtained from suppliers of culture media.

Nutrients that are often depleted quickly include glutamine, arginine, aspartic acid, asparagine and, when growing human diploid cells, cysteine. Amino acid analysis will help determine utilization rates for different cell types. Glucose is used in a wasteful manner by cells, so it is desirable to start at concentrations below 2 g/L and then add more after 2–3 days. An alternative is to switch to other sugars as sources of carbon and energy, for example, fructose or galactose.

7.1.1.3 Amino acids

The requirement for essential amino acids becomes larger when non-essential amino acids are not provided. The beneficial effect of non-essential amino acids is illustrated in Figure 59. There is an extremely rapid utilization of amino acids during the lag phase of growth, and a long lag phase will reduce the maximum cell population when amino acids are growth-limiting (172). Long lag phases are often encountered with primary cells and diploid human fibroblasts, and amino acid depletion can occur at low cell densities and in the absence of exponential growth. In the case of diploid human fibroblasts growing in MEM, the concentration of amino acids becomes division-restricting within 72–96 h after plating (173).

Although no single amino acid may ever reach total depletion, medium replenishment is required. Cystine, glutamine, isoleucine and serine are the amino acids utilized most rapidly, even in microcarrier cultures of diploid human fibroblasts (90). It is usually these that are depleted first by a variety of types of cells.

Deficiencies in the supply of any essential amino acid stresses cultured cells and may inhibit cell division, induce chromosome damage, and increase lysosomal activity and cell size (174, 175). Furthermore, there is a long recovery period after such restriction (175). Restricted amino acid supply is a frequent occurrence with many culture procedures, and limiting concentrations or imbalances in amino acid levels should be avoided if high cell yields are to be achieved. One way of avoiding imbalances or fluctuations in amino acids levels (or other medium components) is to follow a strict medium replenishment scheme (Section 8.4.3.2). It is important to note that additions such as lactalbumin hydrolysate often provide non-physiological mixtures of amino acids and may even result in changes in karyotype (176). Use these complex mixtures with caution when working with many primary cells and cells with low plating efficiencies that have not been adapted to growth in medium containing these supplements. Since large imbalances or excessive concentrations reduce growth, do not supply amino acids at levels that differ widely from the original formulation of the medium. The only exceptions are glutamine and cystine, which may need to be supplied at concentrations different from the original formulation.

Glutamine plays a vital role in metabolism, is a precursor for nucleic acid synthesis, and also an important carbon source. Glutamine is the most unstable amino acid and decomposes in culture medium to form pyrrolidone carboxylic acid and ammonia (177). Regular addition of glutamine will replenish the culture medium and compensate for this decomposition. Increasing the concentration to 2.5 mM during the initial stages of culture usually results in better cell growth, especially at low cell densities (178). Consistent supply of glutamine is also important because of the likely role of this amino acid in the formation of molecules involved in cell-substrate adhesion (179).

The optimum concentration of cystine depends to a large extent on the serum concentration and batch (175). If low concentrations of serum are used, cystine concentration may need to be reduced. For general microcarrier culture, the cystine levels given in the original culture media formulations are adequate, but to optimize a particular process, it may be valuable to examine the effect of different concentrations of cystine. When replenishing culture medium without complete replacement, fresh cystine can be added about every 3–4 days at the concentration stated in the formulation (Section 8.4.3.2, Fig 60).

Fig 60. The depletion of medium components during growth of secondary chicken fibroblasts in microcarrier culture. Cells were cultured in DME supplemented with 15 mM HEPES. 5% (v/v) calf serum, 1% (v/v) chicken serum and 1% (w/v) tryptose phosphate broth and containing Cytodex 1 microcarriers (5 mg/mL). After 3 days the culture medium was replaced by fresh medium ─○─) or was removed and supplemented with cystine (30 µg/mL), glutamine (0.3 mg/mL), inositol (2 µg/mL), glucose (2 mg/mL), choline HCl (1 µg/mL) and 1% (v/v) calf serum, 1% (v/v) chicken serum. The medium was well mixed and returned to the culture (─▲─). Control cultures were not refed (─●─). Replenishment of all the medium was necessary after 7 days if the microcarriers were to be kept confluent. (Clark, J., Hirtenstein, M. Annals N.Y. Acad. Sci. **369**, 33 (1981) by kind permission of the authors and publisher.)

7.1.1.4 Nucleic acid precursors

A supply of components such as adenosine, guanosine, cytidine, uridine (each 10^{-5} M) and thymidine (3×10^{-7} M) is often beneficial, particularly when folic acid is in short supply and when cells are cultured at low densities. Most media contain 1–4 mg/mL folic acid but Medium 199 contains only 1/100 of this amount. In some cases, e.g., primary cells or when culturing normal diploid cells, adding extra folic acid or thymidine may therefore be necessary when using Medium 199 (180).

7.1.1.5 Carbon sources and lactate

Cell growth in culture requires a source of carbon. In most of the commonly used media, this source is provided by glucose (5–20 mM) and glutamine (0.7–5 mM). Glucose is also essential for continued attachment of cells to the microcarriers, and if its concentration falls below approximately 20 mM, cells detach (181). Media containing glucose should be supplemented with pyruvate (1 mM) for growth under low-density conditions. The type of carbon source in the culture medium influences the formation of lactate. The use of different carbon sources in controlling pH is discussed in Section 7.3.3.

7.1.1.6 Vitamins and choline

Adding retinoids can promote the adhesion of cells that otherwise adhere weakly to substrates (182); retinol or retinoic acid can be added at up to 1 mg/mL. Their effect may be due to influencing the synthesis of specific glycoconjugates of the cell surface (182). A sufficient supply of choline is vital to successful microcarrier culture. The main fate of choline is incorporation into membrane phospholipids, and when choline levels decrease, the resulting perturbation of membrane composition causes rounding of cells and decreased adhesion (183). Additional choline is important when using low concentrations of serum (less than 5%). Culture media should not contain ascorbic acid when producing RNA tumor viruses (184). In contrast, ascorbic acid enhances the yield of interferon from microcarrier cultures (102).

7.1.1.7 Polymers

A high molecular weight component may be a necessary supplement for culturing some types of cells at low cell densities (185). When working with cells with very low plating efficiencies (less than 1%), and low inoculation densities (less than 8×10^4 cells/mL), including a polymer in the medium is beneficial during the early stages following inoculation. Polymers improve cell survival but have no effect on growth. Suitable polymers are Ficoll 400, Pluronic F68, Dextran T-70, Dextran T-500 or methylcellulose. A concentration of 1 mg/mL is sufficient.

If urea cycle components and other products of metabolism accumulate to toxic levels at the later stages of the culture cycle and medium replacement is not possible, carboxymethyl cellulose (0.1 mg/mL) can be added to reduce these toxic effects. Ficoll 400 can also be used to reduce turbulence when culturing cell types that attach very weakly to culture surfaces, e.g., some established cell lines such as lymphoblastoid cells.

7.1.1.8 The purpose of serum in culture media

A serum supplement is usually an essential component of media for animal cell culture; in the absence of serum, most cells fail to proliferate. Sera used to supplement culture media come from a variety of sources and are used at concentrations ranging from 0.5% to 30% (v/v). In microcarrier culture, a supplement of 5–10% (v/v) is usual for general-purpose cultures. Whereas culture media are chemically defined, the serum supplement is undefined, especially with respect to those components responsible for promoting growth. The serum serves two vital functions. Firstly, it assists cells attaching to the culture surface, probably by supplying exogenous glycoproteins involved in the attachment process. Secondly, growth factors and hormones in the serum promote cell proliferation. The serum also has a protective effect on cells and enhances viability. A further function of the serum is to provide protease inhibitors that inactivate the trypsin used in routine sub-culturing (Section 8.6.1.2).

7.1.1.9 Choice and concentration of serum supplement

The choice of serum for the growth of a particular type of cell is often based on tradition or convenience. Sera from different species, and even from different batches from the one species, differ widely in their ability to promote attachment and proliferation of cells. Fetal calf serum has been a common supplement because of its high content of fetuin and low content of gamma-globulin and fat (186). This serum often has the ability to promote the growth of more fastidious types of cells.

Fetal calf serum is also unique in having high levels of biotin (187) and, therefore, it provides a source of this growth-promoting component which otherwise is not present in some media formulations (e.g., MEM, DME, L-15). The main disadvantage of fetal calf serum is that it is much more expensive than many other sera and supply is often limited. (Because of this cost, alternative sera are required for microcarrier cultures of several hundred liters where the concentration of fetal calf serum would otherwise be 5–10% (v/v)). A further disadvantage of fetal calf serum is that it is one of the most variable sera with respect to hormone levels (188, Table 14), and that it contains significant levels of arginase, an enzyme that can deplete the medium of the essential amino acid arginine (186).

Table 14. Cell growth and variation of components in batches of fetal calf serum from commercial suppliers. The data are compiled from several sources (188, 189, 190, 191, 192). A measure of variations is provided by CoV (standard deviation/mean × 100).

	Unit	Range	CoV (%)
Plating efficiency*	%	0.6–19.6	52
Cell growth**	10^{-4} cells/cm²	0.57–19.5	66
Protein	g %	1.68–5.30	10
Hemoglobin	mg %	10–110	49
Lactate dehydrogenase	IU	300–3320	36
Gamma globulin	mg %	0–470	111
Total lipids	mg %	140–440	17
Cholesterol	mh %	20–90	24
Free fatty acids	mEq/L	0.1–0.6	43
Uric acid	mg/dL	2.71–11.8	67
Free cortisol	ng/mL	4–34	79
Growth hormone	ng/mL	4.1–167	68
Insulin	mU/mL	0.5–13.7	30
Estrone	pg/mL	11–71	48

*   Based on colony formation of primary hamster embryo cells.
** Based on the number of human fetal lung cells after 72 h.

Figure 61 shows how different sera influence the culture of mammary epithelial cells. Certain sera are good at assisting attachment but poor in promoting cell division. Although the pattern in Figure 62 may not be the same for all types of cells, it illustrates the principle that a variety of different sera should be tested for culturing a particular type of cell. A given type or concentration of serum may not necessarily be optimal for all cell types or all stages of the culture cycle. For example, a high concentration of horse serum (20% or more) favors attachment of mouse glial precursor cells, but a low concentration (5–10%) favors proliferation and differentiation (193). Certain sera may inhibit differentiation of cells in culture. The inhibition of chondrogenesis in chicken limb-bud cells by mouse serum (194) illustrates that some sera have very specific effects. Harrington and Godman (195) describe a factor in the alphaglobulin fraction of some sera that inhibits the proliferation of certain cell lines.

The serum supplement is often the most expensive component of cell culture. Therefore efficient microcarrier culture (particularly for production) requires a flexible approach towards selecting serum supplements. Several different strategies can be adopted, and by selecting one or more of them, the cost of serum supplements can be reduced considerably while still maintaining high cell yields

7.1.1.10 Reducing the serum concentration

Serum supplement concentration can often be reduced below the levels traditionally used for a given type of cell. Maintaining optimal culture conditions (e.g., pH and gas tension) is particularly important if high cell yields are to be obtained in the presence of lower serum concentrations. Giard and Fleischaker (101) reported that 5% (v/v) fetal calf serum was more suitable for microcarrier culture of human fibroblasts than the more usual 10% (v/v) supplement. A period of adaptation or "training" using successive decreases in serum concentrations may improve the growth of cells in reduced serum concentration. Unless special media are used (155), the plating efficiency of cells at low culture densities is proportional to the concentration of the serum, and maximum plating efficiency usually occurs with 10–20% serum. At the beginning of the culture, the role of serum in attachment and protection of cells is important and higher concentrations are often required than at later stages. At higher cell densities, the medium becomes conditioned and cell proliferation depends to a lesser extent on the serum concentration. Hence the requirement for a supplement relates to the function of the serum and depends on the stage of the culture cycle. The concentration need not be constant throughout the culture cycle.

A typical procedure in microcarrier culture is to use a 10% supplement for the first 3 days of culture (or until it contains approximately $1-3 \times 10^5$ cells/mL) and then to use a medium with only 5% serum for replenishment (Section 8.4.3.2). Once the culture has reached confluence, the concentration of serum is reduced further (Section 8.4.3.3), often as low as 0.5%. Horng and McLimans (44, 45) noted that shedding of confluent monolayers could be avoided by decreasing serum concentration. When cultures are used for production of viruses, interferon or other cell products, it is common to omit serum entirely during the production stage. Protein hydrolysates such as lactalbumin hydrolysate or tryptose phosphate broth can to a large extent replace the growth-promoting of serum, particularly when growing established cell lines and certain primary cells. These undefined mixtures of amino acids and polypeptides are often used at a concentration of 0.25–0.5% (w/v).
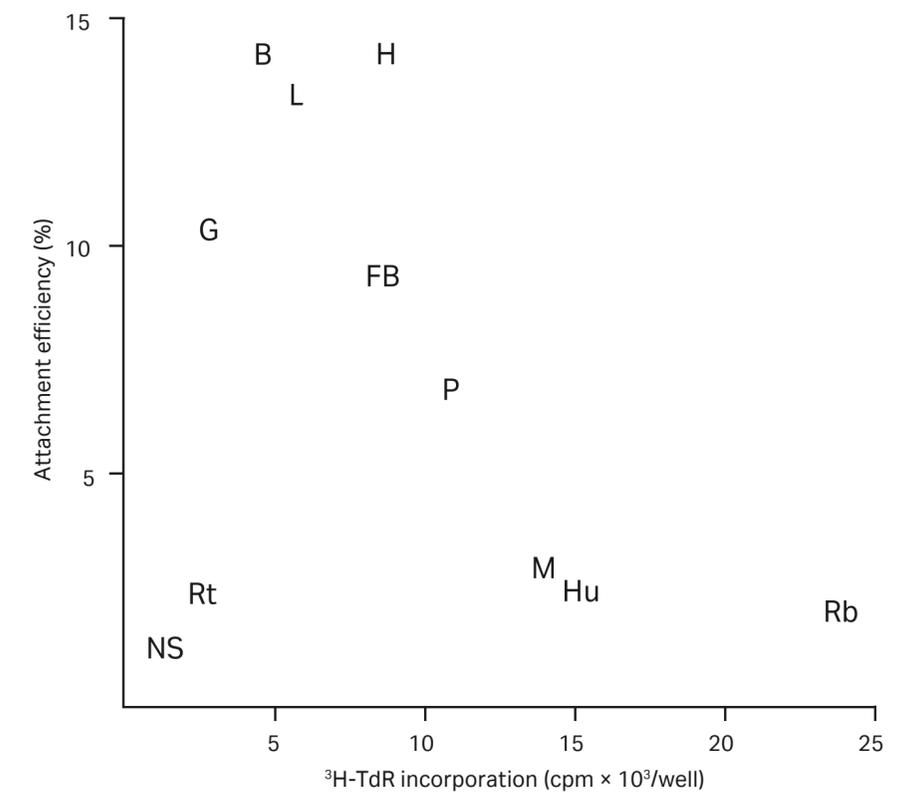
Fig 61. The effect of various sera on the attachment efficiency and thymidine incorporation in cultures of mouse mammary epithelial cells. Cells were cultured in medium supplemented with 20% serum. Abbreviations: B-bovine, L-lamb, H-horse, G-goat, FB-fetal bovine, P-pig, M-mouse, Rt-rat, Hu-human, Rb-rabbit. (Feldman, M., Wong, D., *In Vitro* **13**, 275 (1977) by kind permission of the authors and the publisher.)

7.1.1.11 Changing to another type of serum

In many cases, newborn or donor calf serum can replace the more expensive and less plentiful fetal calf serum supplement. Only a few types of cells require fetal calf serum, e.g., amniotic cells, biopsy material and other primary cultures where cell density is very low. Other types of cells or cultures may show better growth in medium supplemented with fetal calf serum but, after a period of adaptation, acceptable cell yields can often be obtained in other sera. The choice of alternative sera will depend on availability and the scale of the culture, but for larger-scale microcarrier cultures, good quality calf, adult bovine, horse and lamb sera should all be considered as possible alternatives.

7.1.1.12 Blending different sera

Blending different sera can reduce the cost of the serum supplement yet still maintain high yields from microcarrier cultures. A mixture of fetal calf serum and newborn calf serum (50:50) will often result in cell yields identical to those obtained in the same concentration of fetal calf serum (Fig 61). As sera differ in their ability to assist attachment and promote cell division (Fig 61), mixing sera known to support these individual functions can result in improved growth of the culture (196).

7.1.1.13 Different sera for various stages of the culture cycle

Since sera differ in their ability to assist cell attachment and promote proliferation (Fig 61), the best supplement will depend on the stage of the culture cycle. Sera active in promoting attachment and growth under conditions of low cell density can be used during the initial stages of culture. Once the culture enters exponential growth, the growth-promoting function of serum then assumes importance, and sera providing this can be used at the lowest effective concentration. For example, maximum yields of mouse mammary epithelial cells can be obtained by plating in medium containing 20% horse, bovine or lamb serum and then changing to medium containing 5% rabbit serum after 48–72 h (196). Fetal calf serum can be used to stimulate the growth of chicken embryo fibroblasts at low cell densities, whereas horse serum supplemented with 10 mM hemoglobin is more efficient for cultures at high cell densities (197). Figure 62 illustrates that using fetal calf serum for the first 3 days and then changing to medium containing newborn calf serum results in yields of human fibroblasts equal to cultures maintained for the entire period in fetal calf serum. This is a common procedure in microcarrier culture and can be combined with a reduction in serum concentration as the culture proceeds. If the culture is to be maintained for longer periods, the approach of providing a serum supplement is more economical than blending sera.
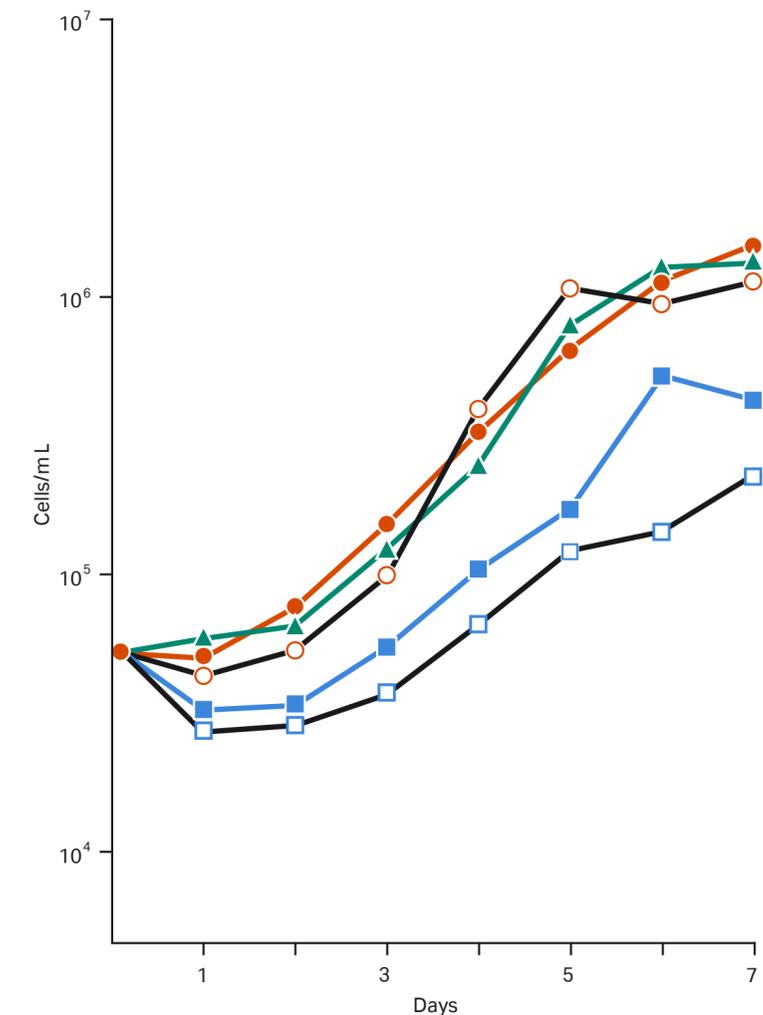
Fig 62. The effect of various types of serum supplement on the growth of monkey kidney cells (Vero) in microcarrier cultures. Cells were cultured in modified DME medium containing 3 mg Cytodex 1/mL and various serum supplements. (—●—) 10% fetal calf serum, (—▲—) 5% fetal calf serum and 5% newborn calf serum, (—○—) 5% fetal calf serum changed to 5% newborn calf serum on day 3, (—■—) 10% newborn calf serum, (—□—)10% horse serum. (Clark, J.M., Hirtenstein, M.D., Annals N.Y. Acad. Sci. **369**, 33 (1981) by kind permission of the authors and publisher.)

7.1.1.14 Variability of sera

One of the most important factors influencing the success and reproducibility of cell culture is the variation between batches of a given type of serum (188, 189, 190, 191, 192). This effect is particularly noticeable with the microcarrier technique where there is often a wide range of culture densities during the growth of a particular culture. The early stages of the microcarrier culture cycle are the most sensitive to variations in the quality of the serum, and when culturing cells with a low plating efficiency (less than approx. 25%), or when starting cultures at a low density (less than 8×10^4 cells/mL with 3 mg Cytodex/mL), a serum supplement of the highest possible quality should be used.

Batches of any given type of serum show large variations with respect to a number of components, and this variation results in widely differing plating efficiencies and yields, even if other culture conditions are optimal for growth (Table 14). A batch of serum can also determine which medium components become limiting. For example, the serum batch is critical in determining the division-limiting concentrations of cystine (175) and variable levels of arginase can rapidly deplete the medium of arginine (186).

Successful cell culture depends to a large extent on using the best batches of serum. Many cases of failure or heterogeneity in microcarrier cultures are associated with poor serum batches. Since microcarrier culture is a method that is directed towards achieving the highest possible yields from a given volume of medium, screen batches of serum whenever possible. Selecting the best batches of serum should be based on plating tests in microcarrier culture. Batches giving maximum cell attachment and growth in Petri dishes are not necessarily the most suitable for microcarrier culture (Table 15). This effect may reflect different affinities of the toxic components in the serum for various culture surfaces. Batches of sera can be screened by simple plating and growth tests on microcarriers in bacteriological Petri dishes. A simple test, modified from that described by Federoff and Hall (193), is to plate 10^4–10^5 cells/mL in HBSS containing 3 mg Cytodex/mL and 50% serum of the batch to be tested. If a large number of cells are still unattached or granular after 24 hrs, the batch is rejected. A further test when screening batches of serum is to determine the attachment efficiency and growth of cells in static cultures containing Cytodex (Table 15). This test uses medium containing 10% serum.

Tests can be performed in parallel with standard plating efficiency tests in monolayer culture (192). Serum is also a potential source of contamination, so use only batches free from microorganisms, including viruses, bacteriophage and mycoplasma.

Table 15. Effect of various batches of fetal calf serum on attachment efficiency of human fibroblasts (MRC-5) in Petri dish and microcarrier cultures

	Attachment (%)					
	Batch 1		Batch 2		Batch 3	
Petri dish	69.2	64.7	61.6	58.3	52.8	50.1
Microcarriers	49.4	53.6	66.1	64.3	55.1	58.6
Yield in microcarrier cultures after 8 days (10^6 cells/mL)	0.5	0.7	1.5	1.0	0.7	1.0

All cultures were inoculated with 5.6×10^3 cells/cm^2. Microcarrier cultures contained 3 mg Cytodex 1/mL and were static in bacteriological Petri dishes. Attachment was determined after 24 h. Results are from independent duplicate experiments. (Clark, J., Hirstenstein, M.D., Gebb, Ch., Develop. Biol. Standard 50, 81–91 (1981), by kind permission of the authors and the publisher.)

7.1.2 Serum-free media

The undefined nature of serum supplements plus their variation in quality makes the use of serum-free media an interesting goal for cell culture. Recent developments in formulating media (198) show that a wide variety of cells can be cultured in the absence of a serum supplement provided that specific components are added to the medium. Certain formulations can support the growth of cells in microcarrier culture (199). Components that can replace serum in microcarrier culture include fibronectin, transferrin, insulin and epidermal growth factor. Most serum-free media formulations are probably suitable for microcarrier culture, but the stirred nature of the culture system means that additional, high molecular weight components such as serum albumin or Ficoll are required to protect the cells (200). Soybean trypsin inhibitor (0.5 mg/mL) should be used when harvesting cells to be cultured in serum-free media.

7.1.2.1 Additives

Many industrial animal cell culture bioprocesses still use serum or other animal-derived proteins as additives for culture media. However, many of these substances are considered a potential risk for the biological safety of the product due to the presence of contaminating agents, such as pathogenic viruses and prions (201, 202). Variable quality and the necessity to eliminate such agents during downstream processing make replacement by safer additives of non-animal origin a priority (Table 16).

This has led to an increasing demand for serum-free (SFM) and protein-free (PFM) media. In many cases, the characteristics of cells cultivated in SFM or PFM will change, and the proliferation, productivity and robustness of the process (203) are influenced. For instance, the adherence of a given cell line may change dramatically, in some cases resulting in a total loss of its ability to adhere to surfaces (204). This can be an advantage if cells are cultivated in suspension mode. Similarly, addition of certain chemicals, such as Pluronic F68, dextran sulfate, PEG or ferric citrate, may also enhance single cell formation (22, 205, 206). To enhance proliferation and productivity, biopolymers or recombinant proteins have to be added to cell culture media. Soy-peptone or other plant-derived hydrolysates can fulfill these requirements (207, 208, 209). Use of either chemically-derived or plant-derived oligopeptides is also considered to increase the yield of bioprocesses (210, 211). An advantage of such media and additives is the known composition of ingredients. Furthermore, transition metals such as V, Cr, Mo, Mn, Fe, Co, Ni, Cu, Zn, as well as fatty acids and vitamins have to be added. Serum normally contains enough of these substances (personal communication with Kathleen Harris at the Cell Culture Engineering Congress, Snowmass, 2002). In the case of metals, the use of standardized "tap water" instead of water-for-injection might solve such problems. Required fatty acids, vitamins and other growth factors have to be first determined and then added to animal cell culture media. Franek *et al*. (212) suggested supplementing protein-free medium with 20 µM ethanolamine, 20 µM ascorbic acid and 500 µM ferric citrate for hybridoma cell lines. Iron is one of the major growth factors for animal cells. Normally, the transport of iron into the cell is mediated by transferrin (213). In protein-free media, transferrin has to be supplemented by different iron salts and iron chelators, such as citrate or EDTA (214, 215).

Table 16. Common media constituents and cell culture reagents derived from animal sources (46)

Constituent	Animal source	Non-animal substitute
Insulin	Bovine/porcine pancreas	Bovine or human recombinant from *E. coli* or yeast
Transferrin	Bovine, porcine or human plasma fraction	Inorganic iron carriers/chelates
Serum protein fractions (e.g., albumin, fetuin, lipoproteins)	Bovine or animal serum	Lipid-delivery alternatives Plant-derived hydrolysates
Protein hydrolysates	Lactalbumin, peptone, casein	Plant-derived hydrolysates
Lipid/sterols	Ovine/human cholesterol piscine lipids, porcine liver	Plant-derived sterols; Synthetic and plant-derived fatty acids
Growth and attachment factors	Murine/bovine organ digests	Recombinant factors Collagen precursors
Amino acids (e.g., tyrosine, cyst(e)ine, hydroxyproline)	Human hair; avian feathers, bovine collagen; bovine/porcine bone gelatin	Synthetic or plant-derived amino acids
Surfactants (e.g., Tween™ 80)	Bovine tallow	Plant-derived polysorbate
Dissociating enzymes (e.g., trypsin)	Porcine pancreas	Plant-derived enzymes Microbial enzymes
Albumin	Bovine	Recombinant in bacteria or yeast

Common additives to low-serum or serum-free media are insulin (IGF-1) (5 mg/L), transferrin (5–35 mg/L), ethanolamine (20 μM) and selenium (5 μg/L). Mixtures of these supplements are commercially available. Media for adherent cells should always contain Ca^{2+} and Mg^{2+} ions as both act as cofactors for adhesion. Sodium carboxymethyl cellulose (0.1%) is sometimes added to prevent mechanical damage to cells. Pluronic F68 (0.1%) reduces foaming and protects cells from bubble shear forces in sparged cultures, especially when low or serum-free media are used. Cyclodextrin or dextrans are also used in serum-free media as albumin replacements.

Some additives have a large influence on the adhesion of cells to microcarriers. For example, pluronic F68 mostly reduces the adherence of cells. It has been shown (216) that a concentration of 50–250 μM ferric citrate in protein-free media supports the attachment of cells to microcarriers, whereas a concentration over 250 μM decreases adherence, especially for CHO cells.

7.1.3 Protein-free media

Protein-free media should not contain any cell proliferation or attachment proteins, or recombinant proteins as media supplements. The advantage over the serum-free media with recombinant proteins is the price because the recombinant proteins as media supplement are still very expensive. Many individuals working in cell culture use plant-derived hydrolysates such as soy peptone from Quest or other suppliers (Section 7.1.2). In this hydrolysate, polypeptides are inside and it is not defined yet when you say "polypeptide" or "protein". Whether you call the medium containing this polypeptide protein-free or serum-free is not important for the regulatory authorities, as you must specify each component regardless of the terminology.

The disadvantage of these hydrolysates is that they are not defined, and you therefore find numerous quality differences between lots. These differences can be seen in cell growth properties, attachment properties and product expression. Soy peptone lots can behave differently for specific cell lines — what is good for CHO cells may not be ideal for Vero cells, for example. Extensive incoming goods control and lot testing are an absolute requirement when using hydrolysates.

7.2 Gas supply

Supplying the correct amounts of O_2 and CO_2 is important for achieving high yields with microcarrier culture. Both O_2 and CO_2 have metabolic functions, and CO_2 is also usually involved in controlling culture pH (Section 7.3). The gas requirements of individual cells are the same whether grown in microcarrier culture or other systems. Unlike static systems, stirred microcarrier cultures have even gas tensions throughout the culture volume, and the possibility of monitoring in the culture gives the opportunity for accurate control. Balin *et al*. (217) observed the beneficial effects of medium movement on reducing micro-environments having different gas tensions. It is important to note that the gas tensions currently in use are often traditional values and may not be optimal for the growth of a particular type of culture. Investigating alternative gas tensions can therefore be valuable when optimizing microcarrier culture conditions.

7.2.1 Gas supply and exchange in microcarrier cultures

The supply of gas to static microcarrier cultures is the same as that for other monolayer techniques. Towards the end of the culture cycle when culture density is high, it may be necessary to direct a stream of gas (usually 95% air:5% CO_2) over the surface of the culture for a few seconds when taking samples or changing the medium. Stirred microcarrier cultures having volumes up to approximately 500 mL–1 liter are usually kept as closed systems. Provided the vessel is not more than 50% full, high yields can be obtained without a continuous supply of new gas (Section 8.4.3, Fig 63). The gas in the headspace is renewed when taking samples or replenishing the medium (Section 8.5.1). The headspace is briefly flushed with gas (15–20 sec of 95% air:5% CO_2 forced through a Pasteur pipette) before the vessel is sealed. This procedure usually supplies sufficient O_2 and CO_2 to satisfy the metabolic requirements of the cells. An alternative is to place the culture vessel with unsealed caps in an incubator with a humid atmosphere (95% r.h.) and a constant supply of 95% air:5% CO_2.

Gas exchange by diffusion between the headspace and the culture is a relatively slow process, and an important function of stirring microcarrier cultures is thus to improve this exchange. Because recently modified vessels for stirred microcarrier cultures operate at lower stirring speeds than the traditional spinner vessels, the rate of gas exchange is reduced. It may therefore be necessary to improve the supply of gas during the final stages of the culture cycle when using stirring speeds of less than 30 rpm or when using culture volumes exceeding 250 mL. The following steps can improve gas supply and may also need to be considered when culturing primary cells or established cell lines, especially when culture densities exceed $2-3 \times 10^6$ cells/mL. (Note that such additional steps are not usually necessary for normal diploid cell strains.)

- Provide a continuous supply of 95% air:CO_2

- Increase gas tensions in the culture headspace, e.g., by using gas mixtures with a higher concentration of O_2

- Increase stirring speed. The increase can be between 25% and 50% but will depend on the cell type and the degree of confluence (Section 8.4.3.1).
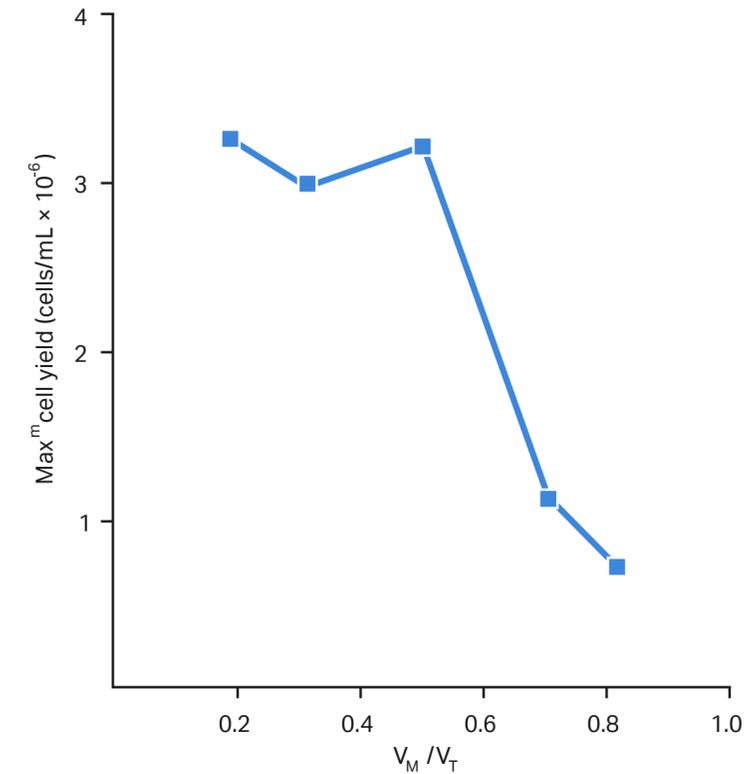
Fig 63. The effect of culture volume and headspace volume on cell yields from a closed microcarrier culture system. Cultures were contained in traditional magnetic spinner vessels (Bellco) with a total internal volume (V_T) of 500 mL. Cultures of various volumes (V_M) were inoculated with Vero cells (8×10^4 cells/mL) and stirred at 50–60 rpm. Cell yield was determined at the plateau stage of the culture cycle (day 8). The culture vessels were sealed and briefly gassed with 95% air:5% CO_2 every day. 50% of the culture medium was changed on day 3 and day 6. (Hirtenstein, M., Clark, J.M., Gebb, Ch., Develop. Biol. Standard **50**, 73–80 (1981), by kind permission of the authors and publisher.)

- Gas the replenishment medium (Section 8.4.3.2) with the appropriate mixture before adding to the culture. This method is a simple way of achieving higher tensions of O_2 in the culture medium.

- Recirculate medium through a gas exchange vessel outside the culture (78)

These steps can also be used to improve gas supply in large-scale microcarrier cultures, normally by improving the supply of O_2 during the later stages of the culture. They should be considered when cell yields are lower than expected, when there is a sudden decline in growth rate or in combination with control of pH (Section 7.3).

Note: Avoid sparging microcarrier cultures. The erratic movement of gas bubbles can damage cells and dislodge them from the microcarriers. Caged sparging separated from microcarriers is a useful solution to avoid dislodging. Direct sparging of macroporous microcarrier cultures is, however, an efficient method for high-density conditions as the cells are protected and held back inside the pores.

7.2.2 Oxygen

Oxygen is a key element for metabolism, and its exact requirement in cell culture depends on the cell type, medium and stage of the culture cycle (218). Static monolayer cultures are relatively anaerobic, and many established cell lines in common use are adapted to such conditions. In these cases, it may not be necessary to have high tensions of O_2 to satisfy the metabolic requirements of the cells.

Primary cultures usually require more aerobic conditions, and the O_2 tension should be similar to that found in the tissue of origin. Oxygen tensions optimal for growth of normal diploid cell strains tend to be intermediate between those required for primary cells and established cell lines. The partial pressure of O_2 in body fluids (pO_2 approx. 95 mm Hg in human arterial plasma) is less than that of air (pO_2 approx. 150 mm Hg at sea level) and although most culture media have been developed for use in approximately 20% O_2, the tension optimal for cell growth is often substantially lower (217, 218, 219, 220, 221, 222, 223). In general, cultures should not be over-gassed since high tensions (above ambient) can be toxic and reduce growth rate. Higher O_2 tensions can also be more toxic at alkaline culture pH (220).

Oxygen tensions affect proliferation rather than cell attachment. Under conditions of low culture densities, low tensions of O_2 (1% to 6%) are optimal for the growth of both normal diploid cell strains and established cell lines (220, 221). During exponential growth, the optimal O_2 tension is usually slightly greater (217, 221, 222, 223, 224). For example, a pO_2 of 9% is optimal for L-cells, and at this tension accumulation of ammonia is at a minimum (152). The optimal pO_2 for growth of diploid human fibroblasts is less than 5% (217). At laboratory scale (up to 1 liter cultures), it is usually sufficient to use 95% air:5% CO_2 as a source of O_2 throughout the culture cycle. The actual tension of O_2 in the medium is lower than in the gas mixture (often 10% to 12%), and provided cultures are not inoculated with very low numbers of cells, a satisfactory plating efficiency will be achieved.

If lower tensions of O_2 are required, e.g., when working with low culture densities (less than 5×10^4 cells/mL with 3 mg Cytodex/mL), the medium can be degassed by vacuum or by flushing with nitrogen. The low tension of O_2 is maintained until the culture density increases to approximately 5×10^4 cells/mL and then 95% air:5% CO_2 can be used. Under low-density conditions, the medium should include HEPES buffer to control pH. When gas tensions can be monitored and controlled, the most appropriate procedure is to start the culture with a low pO_2 (2% to 5%) and increase the tension during the culture cycle to about 15% to 20% at the end of the exponential phase of growth. This increase in pO_2 will also assist in controlling pH (Section 7.3.2). Measure pO_2 tension by immersing a suitable electrode in the medium.

Oxygen tension should be higher (40% to 50%) for high cell density cultures with macroporous carriers to support cells grown in the center of the beads. The right O_2 concentration can be tested by making an oxygen consumption curve versus time. Stop the supply of oxygen to the fermenter and its decrease is proportional to its consumption by the cells. This profile will show a linear decrease up to a certain oxygen tension when the transport of oxygen inside the cells becomes limited due to their mass. At this point the curve flattens. The optimal O_2 tension should be higher than this point.

The solubility of oxygen in aqueous solutions is very low (7.6 µg/mL). The mean oxygen utilization rate of cells has been determined to 6 µg/10^6 cells/h. Oxygen supply depends on the oxygen transfer rate (OTR). OTR = Kla (C*-C). OTR is one of the main limiting factors when scaling up cell culture technology.

Supply is often via surface aeration. This can be increased further by using medium perfusion, increased oxygen pressure, membrane diffusion or by direct sparging of air or oxygen into the culture medium. OTR will increase in vessels with a large height/diameter ratio because of the higher hydrodynamic pressure at the base of the vessel. Membrane diffusion is inconvenient because a lot of tubing is needed, which also makes it expensive. Medium perfusion and oxygenation in a separate vessel have been particularly effective in microcarrier cultures. Sterilizable 0.22 µm non-wettable filters are used to supply gases continuously to cultures.

7.2.3 Carbon dioxide

A solution of CO_2 in the medium results in the formation of HCO_3, an essential ion for growing cells. The requirement for HCO_3 is independent of its buffering action, but since CO_2, HCO_3 and pH are intimately related, it has been difficult to define the tension of CO_2 optimal for cell growth (225). In the mixture 95% air:5% CO_2, the concentration of CO_2 was selected originally on the basis of being the concentration in the alveolar spaces of the lung (225). This concentration was intended for studies on lung fibroblasts but has now become routine for general cell culture. The optimal tension of CO_2 for cell growth may be in the range of 0.5% to 2.0%, with the exact value depending on cell type (225).

To date, most work with microcarrier cultures has involved CO_2 tensions of 5% to 10%, and high cell yields have been obtained. Improved cell yields as a result of lower CO_2 tensions remain to be demonstrated. While it may be difficult to work with lower CO_2 tensions in routine small-scale cultures, the opportunity to control gas tensions and pH in most large-scale systems could be used to define the CO_2 tension that is optimal for growth. Note: Leibovitz L-15 medium does not rely on CO_2 for buffering or control of pH and can be used when low tensions of CO_2 are required. The role of CO_2 in controlling pH is the most important aspect to consider when optimizing conditions for high cell yields. This is discussed in Section 7.3.

7.2.4 Purity of gas supply

All gases used for cell culture should be of the highest possible quality (medical grade if possible). It is important that the supply is essentially free of CO, nitrous oxide and hydrocarbons (225). Use a membrane and/or cotton wool filter to remove any particulate matter in gases introduced directly into cultures. Wide variations in the actual pCO_2 levels can be found in commercial gas mixtures (225), but this variable can be minimized by using certified sources.

7.3 Culture pH

Since pH influences cell survival, attachment, growth and function, maintaining the correct pH is central to obtaining optimal cell growth and high yields. Controlling pH is particularly important when using microcarrier culture because cultures can rapidly become acidic at high cell densities. This decrease in pH is one of the most common causes of poor results in microcarrier culture and is due to accumulation of lactate.

Methods for controlling pH include buffering to minimize the effects of lactate on culture pH (Section 7.1.1.8) or altering culture conditions so that cells produce less lactate (Section 7.3.3). Note: The effect of temperature on pH should always be taken into account and, if possible, pH should be measured at the culture temperature. Unless stated otherwise, all values refer to pH at 37°C.

pH is very important during inoculation. Cell attachment to carriers with an electrostatic surface is highly dependent on the right pH (e.g., a pH of 7.4 is recommended for Vero cells and Cytodex 1 microcarriers). However, medium pH has little effect on cell attachment to coated microcarriers (226).

An alkaline pH prevents/prolongs attachment, and higher pHs kill the cells. The lower setting for pH is normally 7.0. Below 6.8, it inhibits growth. An incubator set point of 5% CO_2 is normally used together with sodium hydrogen carbonate to stabilize pH. Autoclavable probes are normally used in fermenter systems and an upper and lower pH set point chosen. If alkali is needed to compensate for an acidic pH, adding 5.5% $NaHCO_3$ is preferable to 0.2 M NaOH. NaOH can, however, be used in very well mixed systems where it is not delivered directly onto cells. Note that silicone tubing is gas permeable and can cause changes in pH during media transport.

7.3.1 pH optima for cell culture

A pH of 7.2–7.4 is commonly used in cell culture. The wide fluctuations in pH that often occur during the culture cycle and after medium replenishment (pH 7–8) have an adverse effect on cell yields (227). Föhring *et al.* (97) concluded that a constant pH was the most important parameter affecting growth rates and yields of cells and virus in microcarrier culture.

Attachment and plating efficiency depend on the pH of the medium (Fig 64), and one of the most critical stages of culture with respect to pH is just after inoculation (228). To achieve the highest possible plating efficiency, the culture should have a pH of less than 7.6. When initiating a culture, the medium is often exposed to the atmosphere for some minutes, and at the time of inoculation the pH can be as high as 8.0. It is therefore important to ensure that the medium is exposed to the atmosphere for as short a period as possible. In routine microcarrier culture, it is advisable to equilibrate the medium for a few minutes with 95% air:5% CO_2 before inoculation (Section 8.4.2.1). HEPES buffer can also be used to ensure that the medium is not too alkaline during the early stages of the culture cycle (Section 7.3.2).

Diploid human fibroblasts are particularly sensitive to alkaline conditions (pH greater than 7.6) during attachment (Fig 64). When culture pH can be controlled, diploid human fibroblasts should be cultured at pH 7.4–7.5 for the first 1–2 days of culture, after which pH can be increased to 7.6–7.8 for the exponential phase of growth. pH should be decreased to 7.4–7.5 during the plateau stage of culture to ensure continued adhesion of the resting monolayers (Section 8.4.3.3). The pH that is optimal for growth may not be the same as that for attachment. Table 17 shows that by maintaining cultures at a pH optimal for cell growth, substantial increases in yields can be achieved.
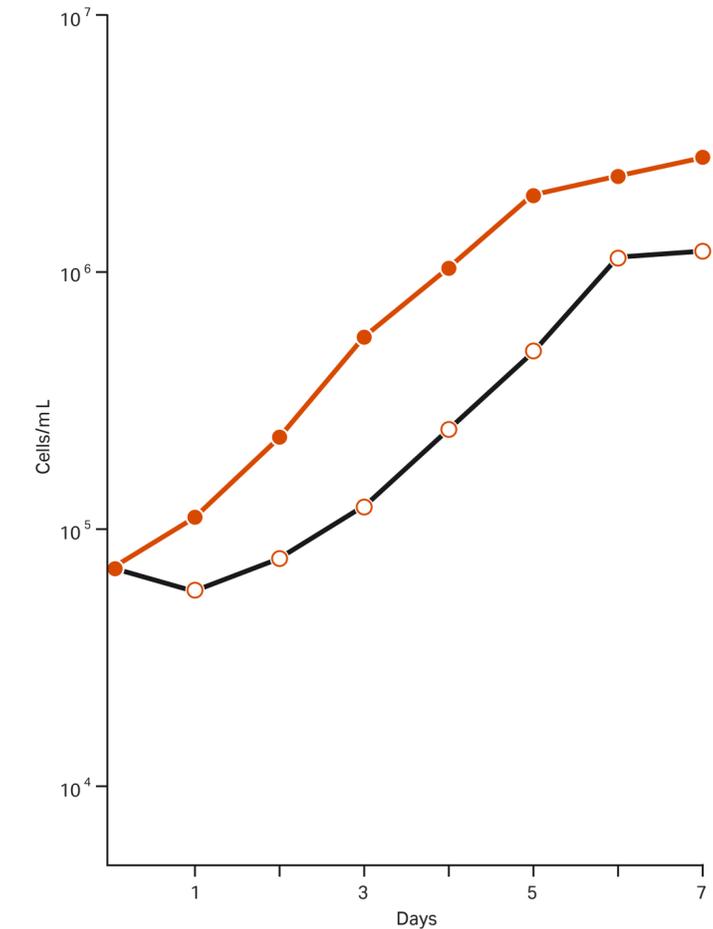
Fig 64. Effect of control of pH on the growth of monkey kidney cells (Vero) in microcarrier cultures. Cultures (400 mL) were contained in sealed spinner vessels and stirred continuously at 50 rpm. Culture medium (DME, based on Earle's salt solution) was with (—●—) or without (—○—) 10 mM HEPES. The pH of the medium without HEPES was initially 7.4 but varied between 7.0 and 8.0 during the culture cycle. The pH of the medium containing HEPES was maintained at 7.3–7.4. The culture medium was changed on day 4. The difference in cell yields was largely due to a higher proportion of empty microcarriers in the culture without pH control. (From Pharmacia Biotech AB, Uppsala, Sweden.)

Different types of cells may have different pH optimal for growth (229, Table 17). By maintaining the pH at a level optimal for growth, serum requirement can be reduced by at least one half (228). In general, human fibroblasts are grown at a higher pH (7.6–7.8) than established cells (pH 7.0–7.4), and it is usual to culture primary cells at a pH of 7.2–7.4. The optimum pH for growth of human foreskin fibroblasts (e.g., FS-4) at low culture densities is more alkaline than the optimum pH for human lung fibroblasts (e.g., MRC-5, 230). When culturing these cells during the growth phase at a density of 10^5 cells/mL or less, the pH should be 7.7–7.8 for FS-4 cells and 7.5–7.6 for MRC-5 cells. For CHO cells, it is normally advantageous to cultivate the cells at pH 7 during attachment. After several hours, you can increase to higher values.

Table 17. The effect of pH on cell growth

Cell type	Strain	pH for optimal growth*	Increased growth at optimal pH[†]
Human			
Normal	KL_2	7.5–7.7[‡]	3.2
	MS2A	7.6	1.9
	Penny	7.5–7.8[‡]	2.1
	WI38	7.8	2.6
SV 40-transformed tumor	WI26VA	7.3–7.5[‡]	1.2
	HeLa	7.0	1.3
Rabbit lens		6.9	3.7
Mouse fibroblasts	3T3	7.5–7.8[‡]	1.8
	L929	7.0–7.5[‡]	1.0

* pH measured at 25°C

[†] Relative to growth in bicarbonate-buffered medium, pH fluctuates from pH 8 to 7 after each refeeding.

[‡] Essentially equal over indicated range.

(Data from Ceccarini, C., and Eagle, H., Proc. Nat. Acad. Sci. US **68**, 229–233 (1971) by kind permission of the authors and publisher.)

7.3.2 Buffers and the control of pH

The buffer system frequently used in cell culture media, carbon dioxide/sodium hydrogen carboxide, is a weak system. At times, it is beneficial to change to a better system. HEPES (10–20 mM) is, therefore, used as an alternative (231), especially in serum-free media where the buffering capacity of the serum needs replacing.

The method used to monitor pH depends on the precision required. Good results with small-scale culture (up to 1L) can be obtained simply by acting on changes in the color of the phenol red indicator in the medium. At larger scale, it is normal to use electrodes to monitor pH. McLimans (232) describes how to control pH when preparing medium. Most media utilize a CO_2/HCO_3 buffer system but its capacity is often not sufficient to prevent pH decreasing towards the end of the culture cycle.

If a cell type produces large amounts of CO_2, media based on Hank's salt solution (0.33 $NaHCO_3$/L) are more suitable than media based on Earle's solution (2.20 g $NaHCO_3$/L). Alternatively, if the cell type tends to produce large quantities of lactate, use formulations with a higher concentration of HCO_3. It is normal to use media containing Earle's salt solution in combination with 95% air:5% CO_2 and media containing Hank's salt solution with lower concentrations of CO_2 in the gas phase. Thus, microcarrier cultures contained in sealed spinner vessels usually require media based on Earle's salt solution.

Adding HEPES delays the onset of pH drift and usually increases cell yield (Fig 65). HEPES assists in maintaining pH during the attachment period of culture and plating efficiency is enhanced. Routine addition of 10–25 mM HEPES is advisable when the best results are required from laboratory-scale microcarrier cultures. Whenever the tension of CO_2 is low (less than 5%), the stability of the HCO_3 system is lower and HEPES should thus be used. The exact amount of HEPES should be no more than that required to maintain the pH, so it is advisable to start with 10 mM. Formulations of buffers designed to give good control of pH at specific values can be found in references 233 and 234.

In large-scale microcarrier cultures, small changes in pH can be controlled by adding HCO_3 or increasing the tension of CO_2. Adding NaOH or HCl will control larger changes. The constant monitoring and control opportunities afforded by large-scale systems mean that HEPES is no longer essential for high cell yields. Culture pH can also be controlled when replenishing with fresh medium (Section 8.4.3.2). Care should be taken not to change the osmolarity of the culture medium when adding buffers for pH control (Section 7.4).
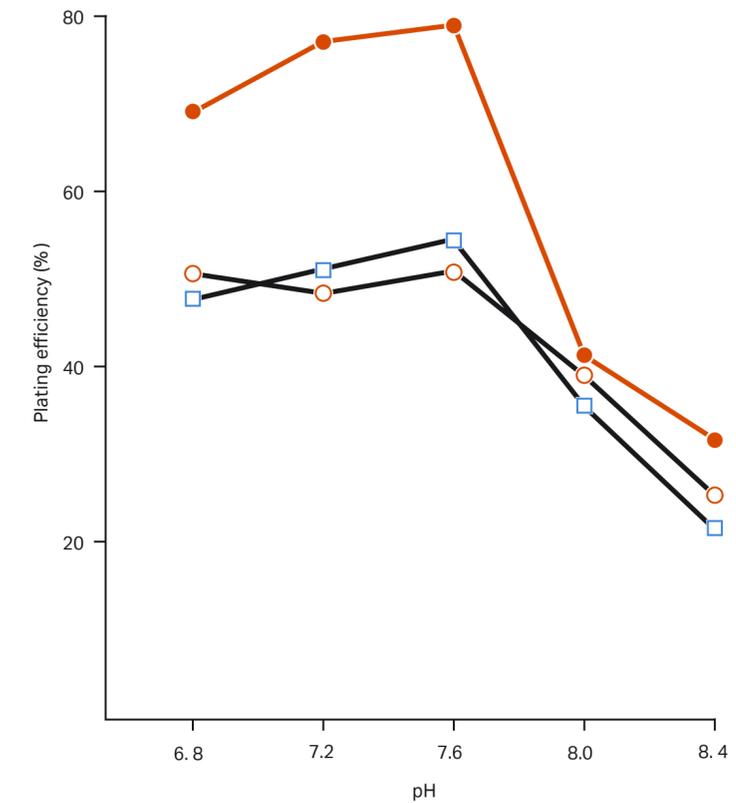
Fig 65. Effect of pH on the plating efficiency of cells. Plating was determined at 12–16 hr by [3]H- thymidine incorporation. (—●—) human embryo lung fibroblasts, (—□—) SV40-transformed W138, (—○—) HeLa. (Ceccarini, C. *In Vitro* **1**, 78 (1975) by kind permission of the authors and the publisher.)

7.3.3 Minimizing accumulation of lactate

One of the most effective ways of avoiding difficulties in controlling a decline in pH is to use culture conditions that limit the formation of lactate. Some established cell lines produce large quantities of lactate and, towards the end of the culture when densities are high, a rapid decrease in pH can occur even in the presence of additional buffer systems. This decrease can reduce cell growth and viability and also detach cells from the microcarriers. A number of methods can limit the production of lactate so that changes in pH remain within the buffering capacity of the medium.

Cultured cells degrade glucose to either CO_2 or lactic acid. Depending on the redox state of the culture, high concentrations of glucose can result in the formation of correspondingly high levels of lactate. When optimizing culture conditions, an effective method of supplying glucose to obtain maximum cell growth is to use 80 µM glucose for the initial stages of culture plus daily replenishment to maintain a concentration of 25–40 µM in the presence of greater than 50 µM glutamine (181). This procedure reduces lactate production and encourages the use of glutamine as a source of carbon. Glutamine is a major source of energy, and the degree of conversion to lactate is less than when glucose is used as the energy source (181, 235, 236).

A maintained or even increased supply of glutamine can also reduce the formation of lactate (222). The amount of lactate secreted by transformed cells can be reduced by biotin (237) and replenishment media can be supplemented with this component. Zielke et al. (181) described a medium that can be used for the high yield cultivation of human fibroblasts with only minimal production of lactate. This medium is based on MEM supplemented with hypoxanthine (100 µM), glycine (100 µM), thymidine (40 µM) and uridine (100 µM).

Increased oxygen tension can be used to reduce lactate formation in the presence of glucose (219, 221). The tension can be increased during the culture cycle and used to encourage a more aerobic metabolism when culture density is high. Oxygen tensions of up to 20% can be used (Section 7.2.2), and an increase to approximately 15% to 20% is particularly useful for reducing lactate accumulation in stationary cultures of human fibroblasts (217).

Another method of reducing lactate is to use a carbohydrate other than glucose. For example, fructose or galactose (2–10 mM) result in greatly reduced levels of lactate (235, 238). When using carbohydrates other than glucose, maintain a good supply of glutamine since this component becomes a major source of energy (235). Increase glutamine levels to 4 mM during the exponential stage of growth when using these alternative carbohydrates.

Changing the carbon source to fructose or galactose is a convenient method for maintaining very dense populations of cells (more than $3-5 \times 10^4$ cells/mL) with the minimum amount of medium. Imamura et al. (238) reported that fructose (5–20 mM) was as effective for maintaining cell growth as glucose (20 mM) in high yield microcarrier cultures, but resulted in virtually no decrease in culture pH. Under identical conditions, diploid human fibroblasts produce only about a quarter as much lactate with fructose or galactose rather than glucose as carbon source (239). Note: Leibovitz L-15 medium contains galactose instead of glucose and can be considered when experimenting with alternative carbon sources. Carbon sources and controlling lactate accumulation should always be considered when optimizing medium replenishment schemes (Section 8.4.3.2).

In summary, useful procedures for avoiding a sudden fall in pH at the later stages of microcarrier culture include improving the supply of glutamine, increasing the oxygen tension and, if necessary, supplementing the medium with biotin. If lactate accumulation is still excessive, use alternative carbon sources. However, these measures need only be taken if changes in pH prove to be too great for the capacity of the buffer systems in the medium.

7.4 Osmolarity

The growth and function of cells in culture depends on maintaining an appropriate osmolarity in the medium (240). Some cells (e.g., HeLa and other established cell lines) can tolerate wide fluctuations in osmolarity. In contrast, primary cells and normal diploid strains are very sensitive to changes in osmolarity, and high yields can only be obtained if it is kept within a narrow range. In the absence of evidence to the contrary, osmolarity used for the culture of any particular type of cell should be kept constant at a value in the range 280–320 mOsm/L, normally 290–300 mOsm/L.

Controlling osmolarity gives more reproducible cultures. Whenever the source of a particular culture medium is changed, osmolarity should be checked. Osmolarity of media produced by commercial suppliers may differ, probably because of differences in interpretation of original formulations (240). Microcarrier culture is no different from any other culture technique in its requirement for a controlled osmolarity. However, high-yield cultures often require various additions to the culture medium during the culture cycle. These additions can include buffers (HEPES), acid (HCl), base (NaOH) and nutrients. Addition of NaCl and the correct amount required to achieve a particular osmolarity is calculated as follows (232): The osmolarity of the medium is measured and the amount of stock NaCl (1 mg/mL) that must be added to achieve the desired osmolarity is calculated.

$$1 \text{ mg NaCl/mL} = 1 \text{ mL stock (mOsm)} = 32 \text{ mOsm increases}$$

$$\text{Hence} \quad \frac{D - O}{32} = X$$

$$\text{where} \quad D \text{ (mOsm)} = \text{desired mOsm}$$
$$O \text{ (mOsm)} = \text{observed mOsm}$$
$$X = \text{mL of stock NaCl (mOsm) to be added per milliliter of medium.}$$

Measuring osmolarity by freezing point depression is the most practical method (240). To not dilute nutrients in the medium, avoid adding large volumes of buffers or saline solutions as much as possible.

7.5 Redox potential

The Redox potential represents the charge of the medium. It is a balance of oxidative and reducing chemicals, pO_2 concentration, and pH. An optimum level for many cells is +75 mV, which equals a pO_2 concentration of 8% to 10% (approx. 50% of air saturation). Redox potential falls under logarithmic growth and is at its lowest 24 hours before the onset of stationary phase (241, 242).

7.6 Storing cells

The most suitable storage medium is either 5% (v/v) dimethylsulfoxide in growth medium or 10% (v/v) glycerol in growth medium. The growth medium should contain a 10% (v/v) heat-inactivated serum supplement.

Cryoprotective agents should be of reagent grade, and accumulation of oxidative products in the stock can be avoided by freezing ampoules of sterilized material. Dimethylsulfoxide is often preferred because it penetrates the cells more rapidly than glycerol. However, the time of exposure of cells to this agent at above freezing temperatures should be as brief as possible. For very sensitive cells, it can be preferable to use a mixture of 5% (v/v) dimethylsulfoxide in fetal calf serum. When preparing the storage medium, mix the cryoprotectant well with the growth medium. Prepare the storage medium immediately before use. It should have a pH of 7.2–7.4.

7.7 Contamination

Preventing contamination by microorganisms is an essential part of all animal cell culture. The risk of contamination can be eliminated by efficient sterilization methods, effective aseptic techniques and antibiotics. Such measures are described in detail elsewhere (156, 157, 243).

The routine use of antibiotics in cell culture media is not recommended because, a) they lead to a relaxation of aseptic techniques, b) resistant microorganisms develop, c) microbial growth may be controlled but biochemical alterations may still be produced, and, d) antibiotics often have adverse effects on cell growth and function. All routine cell culture at Cytiva is performed in the absence of antibiotics.

Antibiotics can depress the growth rate and reduce longevity of animal cells in culture (244, 245). Routine concentrations of penicillin and streptomycin cause at least a 20% reduction in yield from cultures of human fibroblasts compared with the same cells grown for several passages in the absence of antibiotics (245). If microcarrier cultures are to be maintained at confluence for long periods of time, omitting antibiotics reduces any tendency for the cell layer to detach from the microcarriers. Established cell lines may have been selected for growth in antibiotics and may be less sensitive than primary cells (246). Antibiotics can also inhibit protein synthesis in primary cultures (246) and accumulate to high concentrations in fibroblast lysosomes (247).

In certain situations, it may be necessary to use antibiotics. For example, they are required when working with primary cell cultures or cell lines suspected of being contaminated. However, they should be withdrawn as soon as tests show the cells to be free from contamination. Antibiotics may also be necessary for culture systems where there might be a large risk of contamination during the period of culture. In large-scale cultures, the adverse effects of antibiotics are counteracted by the unwanted economic consequences of contamination. Antibiotics are therefore usually necessary for at least the initial stages of the culture production cycle.

During the early stages of culture, samples of medium can be taken for sterility tests. If the medium is sterile when the culture approaches confluence, antibiotics can be withdrawn from the replenishment medium, or at least the concentration reduced. The most suitable antibiotics to use as antibacterial agents are penicillin (100 U/mL), streptomycin (100 µg/mL) or gentamycin (50 µg/mL). Nystatin (50 µg/mL) will eliminate growth of fungi and yeasts. These antibiotics can be used individually or in combination.

The cytotoxicity of antibiotics increases in low-serum and serum-free media, and their quantity should also be reduced in proportion to the serum concentration. Cell cultures must be checked frequently for mycoplasma. Mycoplasma have a wide range of effects on cultured cell growth and function (248) and, most importantly for microcarrier culture, mycoplasma compete with cells for nutrients in the culture medium.

Mycoplasma contamination can rapidly deplete the essential amino acid arginine from the culture medium and increase accumulation of ammonia (248). It is this depletion and imbalance in amino acid composition that has serious consequences for cell cultures. Extensive mycoplasma infection usually leads to failure of the microcarrier culture. High-density animal cell cultures need all nutrients available in the medium, and they will inevitably suffer if they must compete with mycoplasma.

The simplest and most effective method for routine screening of mycoplasma is to use the following fluorescent staining method (249, 250). Cells to be tested are grown on glass coverslips in 5 cm Petri dishes until approximately 70% confluent. Without removing all of the culture medium, 2 mL of modified Carnoy's fixative (3:1 absolute methanol:glacial acetic acid) are added gently to the dish. After 2 min at room temperature, the fixative is replaced by fresh fixative for 5 min. The coverslips are then rinsed briefly in fresh fixative and air-dried. A stock solution (0.05 mg/mL) of the benzimidazole fluorochrome Hoechst 33258 (American Hoechst, Somerville, NJ, USA or Riedel-De Haen AG, Seelze-Hannover, Germany) is prepared in HBSS without phenol red, pH 7.0. This solution is diluted to 0.05 µg/mL with HBSS and the coverslips washed three times in distilled water before mounting in 0.1 M acetate buffer, pH 5.5. A fluorescence microscope is used to check the presence of fluorescent particles at the periphery of the cells. Suitable filters are Zeiss 53/44 barrier filter and a BG-excitation filter. Infected cultures should be discarded. In exceptional cases, measures may be taken to try to eliminate the mycoplasma (158, 243, 248).

7.8 Stirring

Avoid stirrers with moving parts. They will damage both cells and microcarriers. Top-driven reactors are best if the carriers/cells are not physically separated from the stirrer. The geometry and speed of the impeller greatly influence OTR. With certain vessel designs and a marine impeller, 150 rpm has been achieved without being detrimental to the cells.

7.9 Control and feeding strategies

For good final product quality, it is important to have as steady state conditions as possible. Variations in sugar concentration will invariably affect final product glycosylation, for example.

In simple cell culture setups, carrier samples are taken, cells counted and morphology examined (photographs taken) to document growth. As cells grow, stirrer speed is increased to compensate for the increased weight of the carriers (empty carriers 1.03 g/mL, cells 1.015–1.070 g/mL depending on cell type). This keeps them suspended and increases gas transfer. After about 3 days in culture, the medium turns acidic and needs to be changed.

Media supply is determined empirically in relation to growth and productivity, or by amino acid, glucose or lactic acid analysis. In bioreactors, there is continuous control of pH, dO_2 and stirrer speed, usually via a programmable controller. pH is normally initially controlled by adding CO_2 to lower pH. Nitrogen will wash out CO_2 and increase pH. Adding alkali via sodium hydrogen carbonate or NaOH solutions will increase pH during very high lactic acid and CO_2 production.

dO_2 is controlled by adding air or, at high cell concentrations, pure oxygen. Increasing stirrer speed can be coupled to the increased demand for oxygen supply. This increases OTR. To avoid toxic effects when sparging with oxygen, the cells need to be protected from the gas bubbles.

Media utilization is normally determined by taking samples to measure glucose concentration. Sampling is either done manually or automatically via a flow injection analysis biosensor system connected on-line to the bioreactor (251, 252) (Fig 66). These results are then used to determine the rate of manual media change or to automatically regulate the perfusion rate via pump speed. In addition to glucose, glutamine and even metabolites such as lactate can be used to measure media utilization. Ammonia can be measured on-line with the FIA biosensor system. Oxygen consumption rate can also be used to determine cell growth and, when optimized and correlated with the metabolic rates of the substrates, as a parameter for deciding feeding strategies. Switching off oxygen supply and looking at its linear consumption over time allows the number of cells to be calculated.
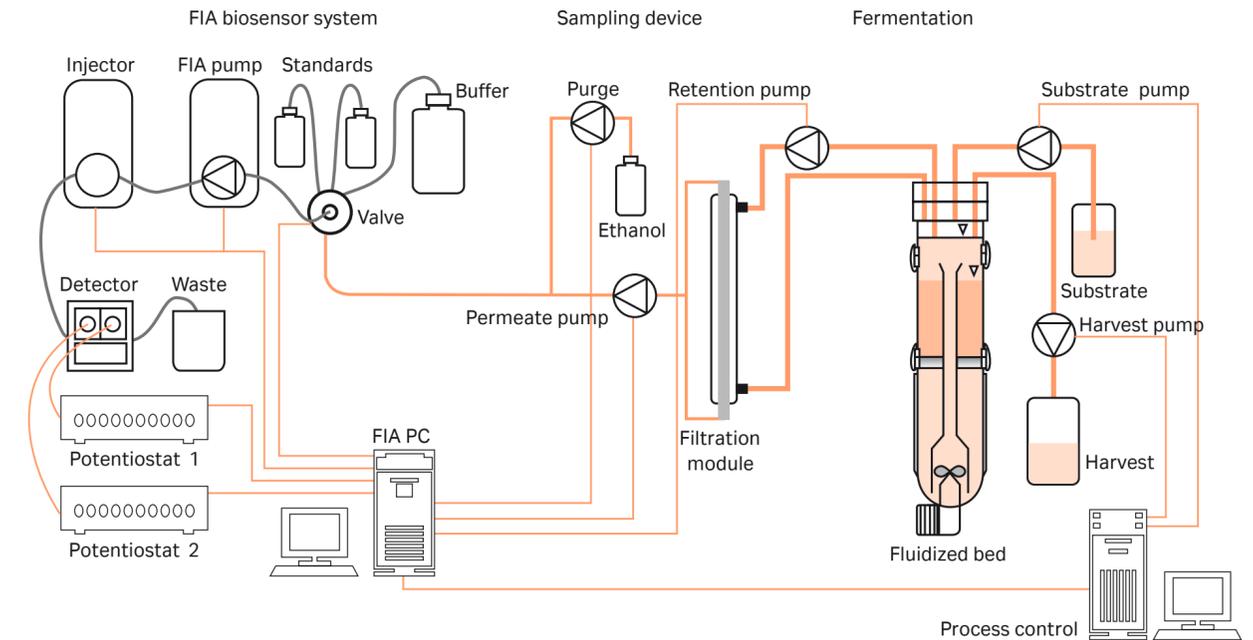
Fig 66. Flow injection analysis (FIA) setup (251).

08
Microcarriers in practice

8.1 General considerations

It is a big advantage if the carriers are supplied dry. The exact amount needed can be weighed and then prepared *in situ* by autoclaving. If a mistake is made during preparation, the carriers can be re-sterilized. Pre-sterilized microcarriers always have to be handled under sterile conditions and can normally not be re-sterilized by autoclaving.

At large-scale, it is advantageous to prepare the carriers directly inside the reactor because of the problem of heat transfer into vessels standing in autoclaves. The correct amount of carrier is added to an already cleaned and sterilized reactor. A volume of distilled water large enough for the microcarriers to swell properly (hydrated microporous microcarriers) and be covered after settling is then added to the fermenter. (Note that for soft microcarriers, the swelling factor in water is much larger than in PBS!) The reactor is then closed and the vessel re-sterilized while the carriers are stirred.

The culture medium is made up from powder, allowing for the water volume remaining inside the reactor. It is then sterile filtered, added to the reactor and all parameters equilibrated. The reactor is then ready for inoculation.

This method works well with serum-containing media. Caution is required when working with serum-free media, where additional washing, sedimentation and decanting of the washing buffer may be necessary prior to sterilization.

Carriers for packed or fluidized beds are also normally prepared inside the reactor. At small-scale, the entire reactor can be sterilized by autoclaving. At larger scale, carriers are sterilized *in situ*.

8.2 Microcarrier concentrations

In homogeneous stirred tanks, the concentration of surface microcarriers in batch cultures is normally 2–3 g/L. However, this can be increased up to 15–20 g/L (surface area of 60–80 000 cm^2/L) when perfusing the culture. In fluidized beds, 10–50% of the working volume is filled with carriers. Fluidization is normally better at higher concentrations. In packed beds, the cage or column is normally filled with microcarriers. The amount is related to the size of the cage or column.

8.3 Inoculum

It is essential to equilibrate and stabilize all culture parameters before adding the inoculum. Temperature and pH are especially important.

When inoculating, use actively growing cells in logarithmic growth. Avoid cells in stationary phase. If the cells are stationary, i.e., in G0, they have to be pushed back into the cell cycle with growth factors. This is normally a 20–24 hour process, which leads to an initial lag phase in growth. Stationary phase cells can lose one day of process time at each subculture step.

The cells used for inoculation should be single cells (Fig 67), and definitely not large aggregates, which will otherwise cause heterogeneity.
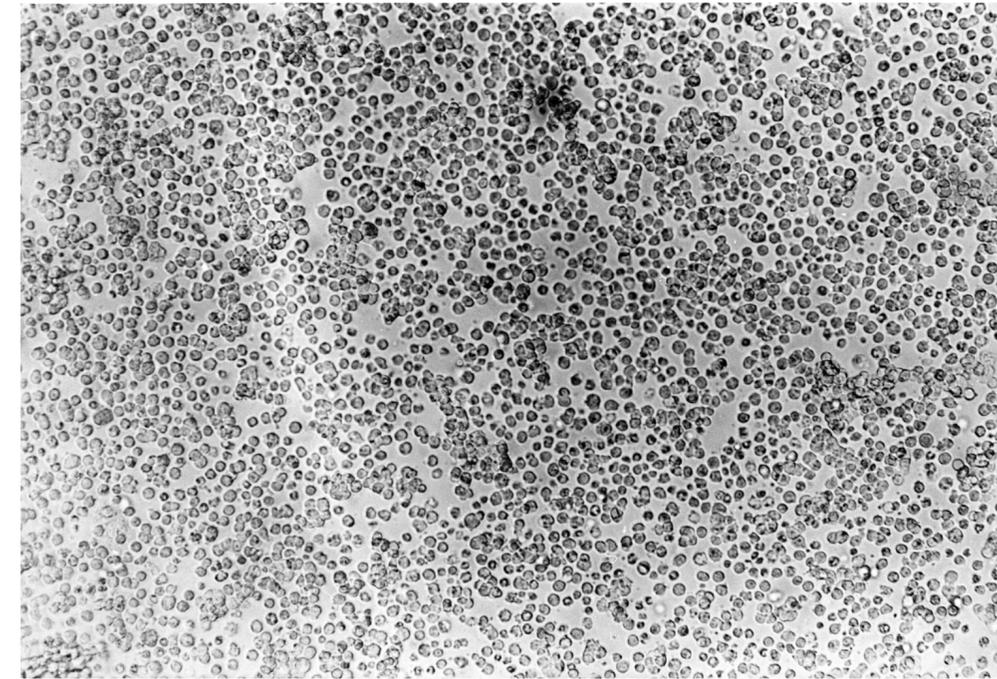
Fig 67. Single-cell suspension.

Surface carriers are normally inoculated with 0.5–2 × 10^5 cells/mL or 0.5–2 × 10^4 cells/cm^2. Macroporous carriers are normally inoculated with about the same cell concentrations. Inoculate the cells in 1/3–1/2 of the final volume. Stir immediately at the lowest speed to keep the micro-carriers in homogeneous suspension. After attachment, add media to the final volume. If the vessel is very well suited for microcarriers, it may be possible to start directly with the final volume.

In some processes, large volumes of cells are generated on microcarriers and then harvested and frozen. These cells are later thawed and used as inoculum straightaway. The process is then scaled up two more steps before final production. This makes production more flexible.

Fluidized beds are inoculated with 1–2 × 10^6 cells/mL of packed bed carrier volume. The inoculation density is highly dependent on both cell line characteristics and media composition. In general, the higher the inoculation density the earlier the production cell density is reached. Stirring rate is also related to the shear sensitivity of the cell line. Normal stirring rates are between 20 and 100 rpm in stirred tank, in the Cytopilot reactor between 100 and 300 rpm. Stirring speed is initially low and increased as the cell concentration increases. During inoculation, the fluidized bed is run as a packed bed. After about 5 hours, the bed can be fluidized.

Consider from the beginning how to create the large quantities of cells needed to inoculate a production-scale fluidized bed. Imagine you need 1 kg of product and your anchorage-dependent cell line produces 100 mg per liter carrier and day. A fluidized bed with 100 liters of carriers has to be operated over a period of 100 days. Some 2 × 10^{11} cells would be needed to inoculate these carriers (2 × 10^6 per mL of carrier). This would require 2000 roller bottles of 850 cm^2.

The problem could be overcome by:

• Preparing the inoculum as aggregates in a stirred tank

• Scaling up on the same carriers via trypsinization

• Scaling up on different carriers (smooth carriers to macroporous)

• Carrier-to-carrier transfer

For suspension cells, the easiest way is always to create the inoculum in stirred tanks before transfer to macroporous carriers. Different inoculation strategies are shown in Figure 68 in comparison to a roller bottle production for a typical CHO cell line.
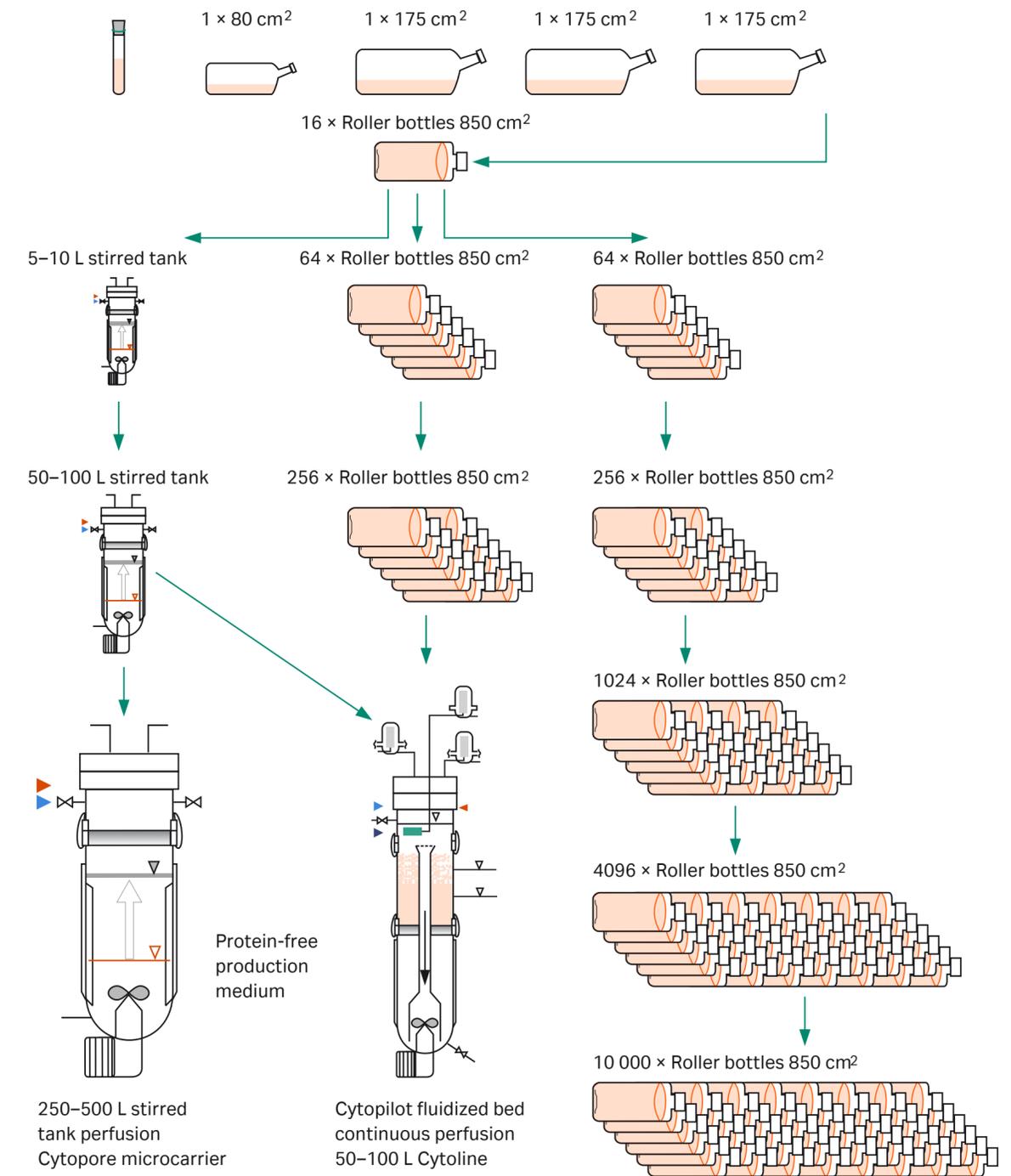
Fig 68. Different strategies of inoculation of large-scale macroporous microcarrier fermentation in comparison to a roller bottle production (split ratio is 1:4).

Figure 69 shows that the attachment phase after inoculation is finished after 2 hours on macroporous carriers in fluidized bed applications. Fluidization can thus be started very soon after introducing the cells into the reactor.
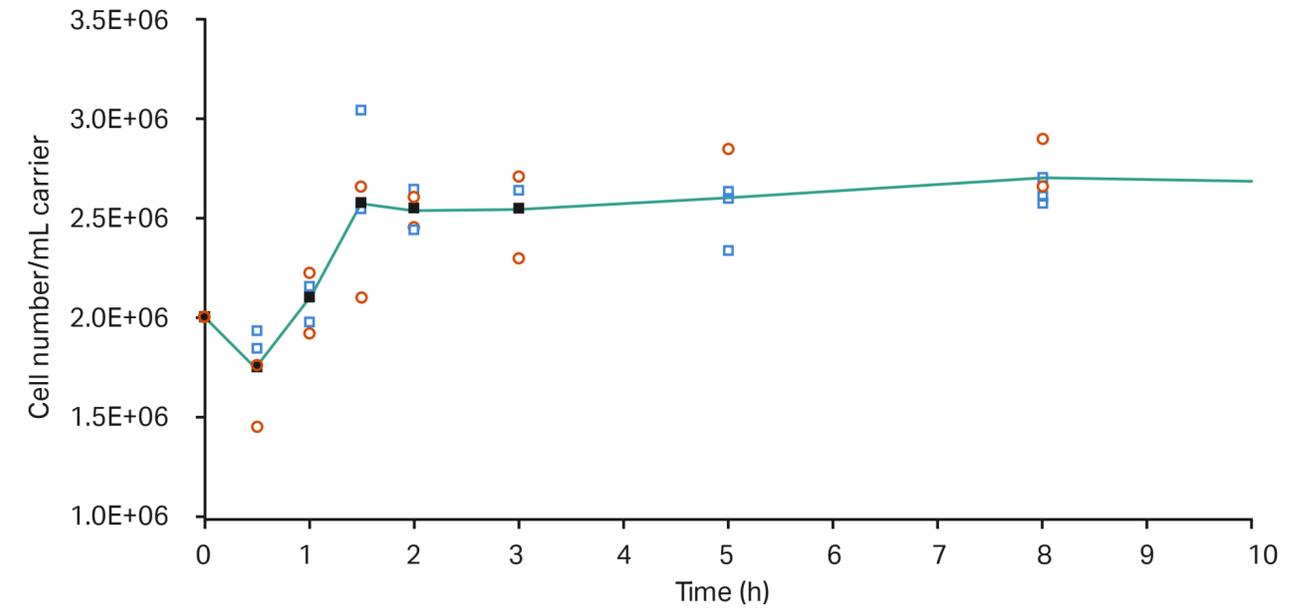
Fig 69. Cell attachment kinetics in fluidized bed culture. The symbols are the experimental data from parallel experiments, and the line is the mean value.

8.4 Preparing microcarriers for culture

8.4.1 Preparing for sterilization

8.4.1.1 Cytodex

Dry Cytodex microcarriers are added to a suitably siliconized glass bottle (Section 6.2.1) and swollen in Ca^{2+}, Mg^{2+}-free PBS (50–100 mL/g Cytodex) for at least 3 h at room temperature with occasional gentle agitation. The hydration process can be accelerated by using a higher temperature, e.g., 37°C. The supernatant is decanted and the microcarriers are washed once with gentle agitation for a few minutes in fresh Ca^{2+}, Mg^{2+}-free PBS (30–50 mL/g Cytodex). The PBS is discarded and replaced with fresh Ca^{2+}, Mg^{2+}-free PBS (30–50 mL/g Cytodex) and the microcarriers are sterilized by autoclaving with steam from purified water. When hydrating Cytodex 3, initial surface tension may occasionally prevent wetting and sedimentation of the microcarriers. Should this occur, add Tween 80 to the PBS used for the first hydration rinse (2–3 drops, Tween 80/100 mL PBS). Alternatively, autoclave followed by rinsing and finally autoclave to sterility. Note: Cytodex 3 does not swell to the same extent as Cytodex 1.

Prior to use, the sterilized microcarriers are allowed to settle, the supernatant decanted and the microcarriers rinsed in warm culture medium (20–50 mL/g Cytodex). This rinse reduces dilution of the culture medium by PBS trapped between and within the microcarriers (a step of particular importance when using small culture volumes or cells with low plating efficiencies). The micro-carriers are then allowed to settle, the supernatant removed and the microcarriers resuspended in a small volume of culture medium and transferred to the culture vessel. It is not necessary to treat the microcarriers with serum or to have serum in the rinsing medium.

8.4.1.2 Cytopore

Hydrate and swell the dry microcarriers in PBS (50–100 mL/g of Cytopore) in a siliconized bottle. Some of the carriers might float at first, but autoclaving will expel any air trapped in the carriers and allow them to settle. Gently agitate the solution occasionally for approximately 10 min at room temperature. Adjust pH to 7–7.5 and then autoclave the microcarriers at 121°C for 20 min. After autoclaving, allow the sterilized microcarriers to settle, remove the supernatant and add fresh PBS. A second wash with PBS is recommended.

Cytopore is very stable and even three autoclavings will not affect cell culture performance. The carriers can be sanitized with 70% ethanol. They also withstand 1% NaOH solution.

8.4.1.3 Other sterilization methods

It is also possible to sterilize the microcarriers by other methods. After swelling the microcarriers in Ca^{2+}, Mg^{2+}-free PBS, let them settle, decant the supernatant and replace by 70% (v/v) ethanol in distilled water. The microcarriers are washed twice with this ethanol solution and then incubated overnight in 70% (v/v) ethanol (50–100 mL/g Cytodex). The ethanol solution is removed and the microcarriers are rinsed three times in sterile Ca^{2+}, Mg^{2+}-free PBS (50 mL/g Cytodex) and once in culture medium (20–50 mL/g Cytodex) before use.

Cytodex 1 can also be sterilized with gamma irradiation (2.5 megarads). The sterilizing step is performed with dry microcarriers before swelling in sterile PBS using the procedure described above.

For large-scale cultures, the microcarriers can be swollen and sterilized *in situ* in fermenter vessels possessing an in-line steam sterilization system. This simplifies dispensing the microcarriers into the fermenter and reduces the risk of contamination.

The above ranges of solute volume to weight of microcarrier allow for different types of cells. For cells with low plating efficiency, use the larger solute volume/microcarrier weight when swelling and sterilizing. Conversely, microcarriers to be used with cells with high plating efficiency can be prepared in the minimal quantities of solute. A reduction in culture medium pH on addition of the microcarriers indicates that hydration and equilibration are not complete. If this decrease in pH is observed, rinse the microcarriers in the medium once more.

8.4.2 Initiating a microcarrier culture

The initial phase of a microcarrier culture is usually the most critical stage in the culture cycle (77). Success depends on following correct procedures when starting the culture and during early growth. Furthermore, the exact procedure is different for each type of cell and will depend on its growth properties in culture. Growth properties such as the rate and strength of attachment to culture surfaces and plating efficiency must be taken into account when selecting inoculation conditions and stirring speed. When initiating a microcarrier culture, always consider the points below. In most cases, the optimal conditions for a given cell type can be deduced from what is already known about its growth in culture (e.g., in Petri dishes or roller bottles) and also from preliminary experiments with microcarriers in Petri dishes (Section 6.1).

8.4.2.1 Equilibration before inoculation

Conditions for attachment should be optimal from the moment the cells are inoculated in the culture. Avoid a long period of equilibration after the culture has been inoculated. Ensuring that the culture is equilibrated before inoculation helps achieve the maximum possible plating efficiency of cells in the inoculum.

Remove the PBS in the sterile microcarriers by rinsing in warm culture medium (Section 8.4). Adjust the culture temperature to a level optimal for cell attachment. In practice, this temperature is usually the same as that of the growth stage of culture (normally 35–37°C). Make every attempt to ensure that the culture pH is within the limits optimal for cell attachment (usually pH 7.0–7.4, Section 7.3). Gas mixtures used during the initial stage of culture (Section 7.2) should be allowed to exchange with the culture medium before inoculation. These factors are particularly important at large culture volumes (more than 500 mL) when it takes longer for equilibration. Small culture volumes (500 mL or less) can be equilibrated by incubating the culture vessel containing medium at 37°C and in an atmosphere of 95%:5% CO_2 (Section 7.2). After a few minutes, the culture will be ready for inoculation. Stirring can hasten the process of gas exchange. Equilibration of cultures with very large volumes may take 2–3 hours. Overnight equilibration is recommended. This long equilibration time can also be used as a short sterility test. Always note the exact procedure used for equilibration if you want to obtain reproducible results.

8.4.2.2 Initial stirring

The key to achieving maximum yields from microcarrier cultures is to ensure that all microcarriers are inoculated with cells from the very beginning of the culture. Transfer of cells from one microcarrier to another occurs only infrequently during the culture cycle and it is therefore important to ensure that the maximum possible number of cells from the inoculum attach to the microcarriers.

One way of initiating a microcarrier culture is to inoculate the cells into the final volume of medium containing microcarriers and immediately begin stirring. Figure 70 illustrates that under such conditions, different types of cells attach to the microcarriers at different rates. The rate and proportion of cells attaching to the microcarriers can be increased if the culture remains static with gentle intermittent stirring during the early attachment stage. If the
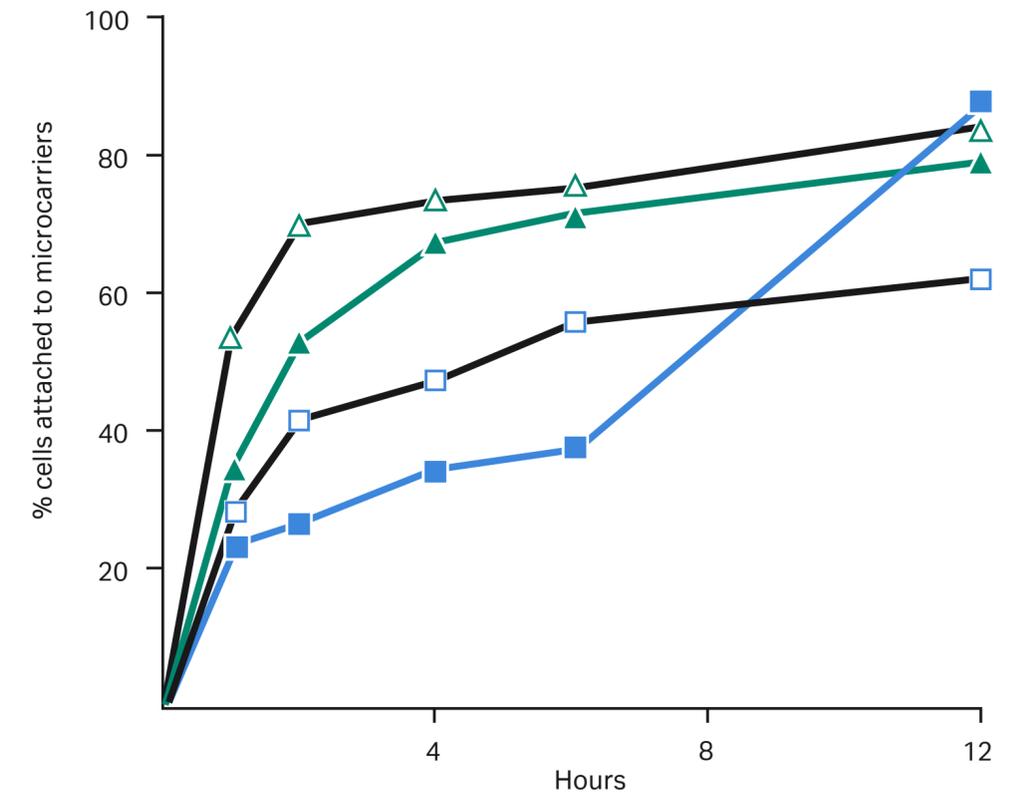
Fig 70. The rate of attachment of various types of cells to Cytodex 1 microcarriers. All cultures were inoculated with 10^5 cells/mL and contained 5 mg Cytodex 1/mL. Stirring was continuous (50 rpm). (—△—) chicken fibroblasts, (—▲—) monkey cells (Vero), (—□—) human fibroblast (MRC-5), (—■—) mouse fibroblasts (L-929). (From Pharmacia Fine Chemicals AB, Uppsala, Sweden.)

cell-microcarrier mixture is contained in a reduced volume (e.g., in 1/3 of the final volume) at the same time as intermittent stirring, cells have a greater chance of coming into contact with a microcarrier, and the conditioning effects on the medium are also much greater.

The ability of an anchorage-dependent cell to attach to a culture surface is reduced if the cell is kept in free suspension for increasing lengths of time.

For cells with an intrinsically slow rate of attachment, culture conditions should allow the cells to attach as rapidly as possible. Figure 71 shows the effect of using an attachment period with intermittent stirring and reduced initial culture volume on the rate of attachment of human fibroblasts and monkey kidney cells. Both procedures use the same total number of cells and microcarriers for each cell type, but the modified procedure leads to more efficient utilization of the inoculum. This procedure results in attachment efficiencies comparable to those observed in plastic Petri dishes.
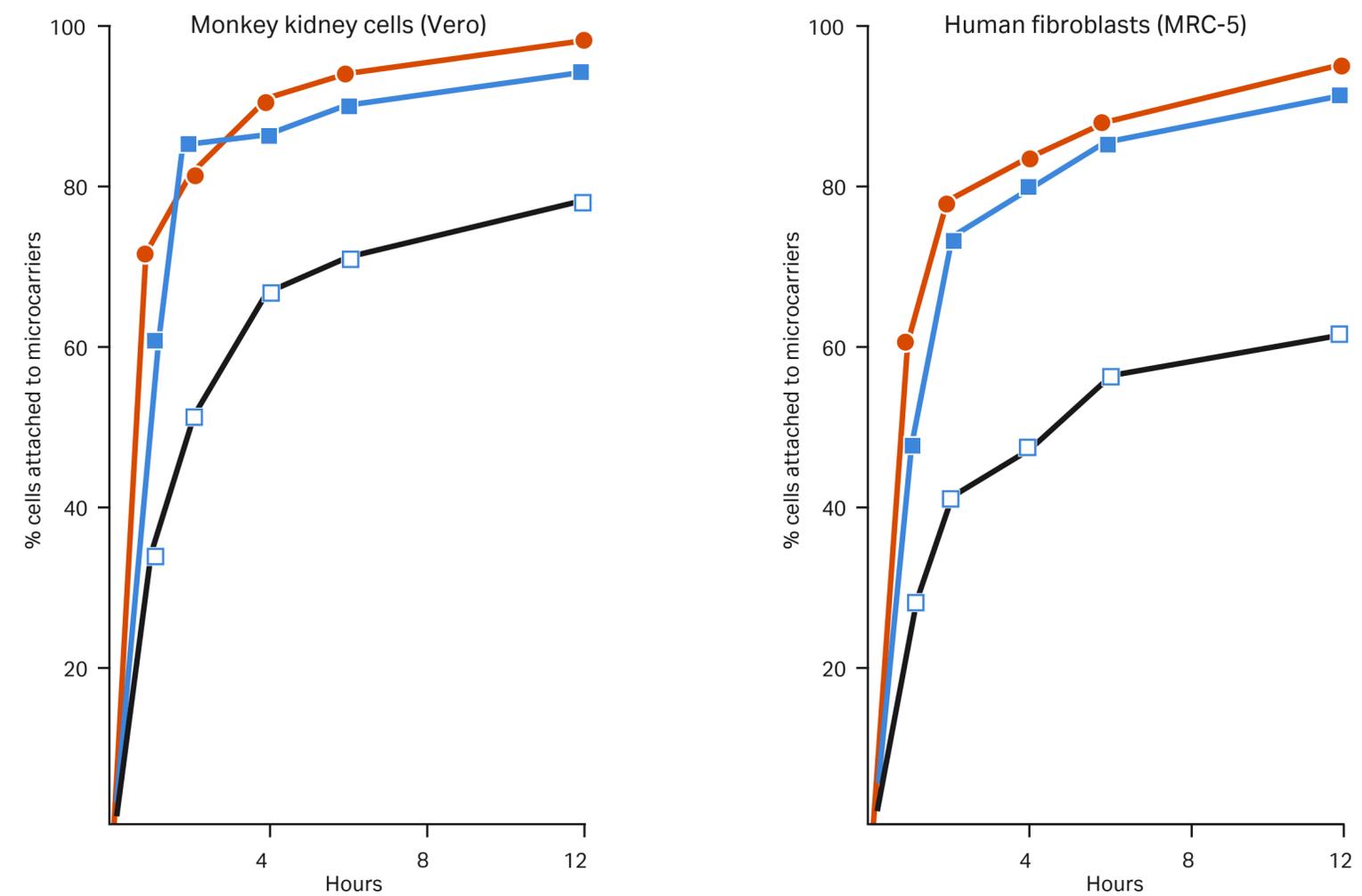
Fig 71. The effect of initial culture procedure on the attachment efficiency of cells. Cultures containing 5 mg Cytodex 1/mL final volume were inoculated with 10^5 cells/mL final volume and were either stirred immediately (—□—, 60 rpm) or cultured in a reduced culture volume with intermittent stirring(—■—). The modified initial culture procedure (—■—) involved stirring for 1 min (30 rpm) every 45 min in 1/3 of the final culture volume. After 3 h the culture was diluted to the final volume and stirred (60 rpm). Attachment of cells to plastic Petri dishes is indicated (—●—). (From Pharmacia Biotech AB, Uppsala, Sweden.)

The increased efficiency of attachment that results from initiating the culture in a reduced volume and with intermittent stirring produces an increase in cell yield at the plateau stage of culture (Fig 72). Improvements in cell yield when using this technique are most apparent where cultures are started with low inoculation densities. This modified procedure can be used for all types of cells and is particularly recommended when working with cells with low plating efficiency (e.g., primary cell cultures and normal diploid cell strains), or if sufficient cells are not available to start the culture with optimum inoculation densities (Section 8.4.2.4).

The specific details of a modified initial culture procedure depend on the cell being cultured. A preliminary experiment with a stationary microcarrier culture in a bacteriological Petri dish will help estimate the time required for attachment to the microcarriers and will also reveal any tendency for cells and microcarriers to aggregate under static culture conditions (Section 6.1).

When starting most cultures, cells and microcarriers are incubated in 1/3 of the final volume. The culture is stirred for 2 min every 30 min at the speed used during the growth phase of the culture (Section 8.4.2.1). After 3–6 h, continuous stirring is commenced at a speed just sufficient to keep the microcarriers in suspension (Section 8.4.3.1), and the volume of the culture is increased with pre-warmed (37°C) culture medium. The longer attachment time is usually used for primary cells with an epithelial morphology. For cells with low plating efficiency (less than 10%), or for cultures started with sub-optimal numbers of cells (Section 8.4.2.6), the culture volume can be maintained at 50% of the final volume for the first 3 days, after which fresh medium is added to the final volume. In this way, the population of cells can be cultured at greater densities during the period the culture is most susceptible to dilution.

Slow continuous stirring during the attachment stage is necessary for cell types that tend to clump when allowed to settle (e.g., primary chicken embryo fibroblasts). In such cases, the initial stirring speed need only be about 25% of that normally used for the growth phase (Section 8.4.3.1). Griffiths *et al*. (7) and Moser and Stoffels (115) have observed that the modified initial culture procedure was essential for good results when growing human fibroblasts and heart muscle cells respectively.

For suspension cells inoculated on macroporous microcarriers, a reduced inoculation volume is essential if the cells are to have a chance of being entrapped inside the pores. In fluidized beds, reduce the initial stirring so that the carrier bed is just expanded and not fluidized. This expanded bed acts like a dead-end filter unit where all the cells are filtered out of the medium.
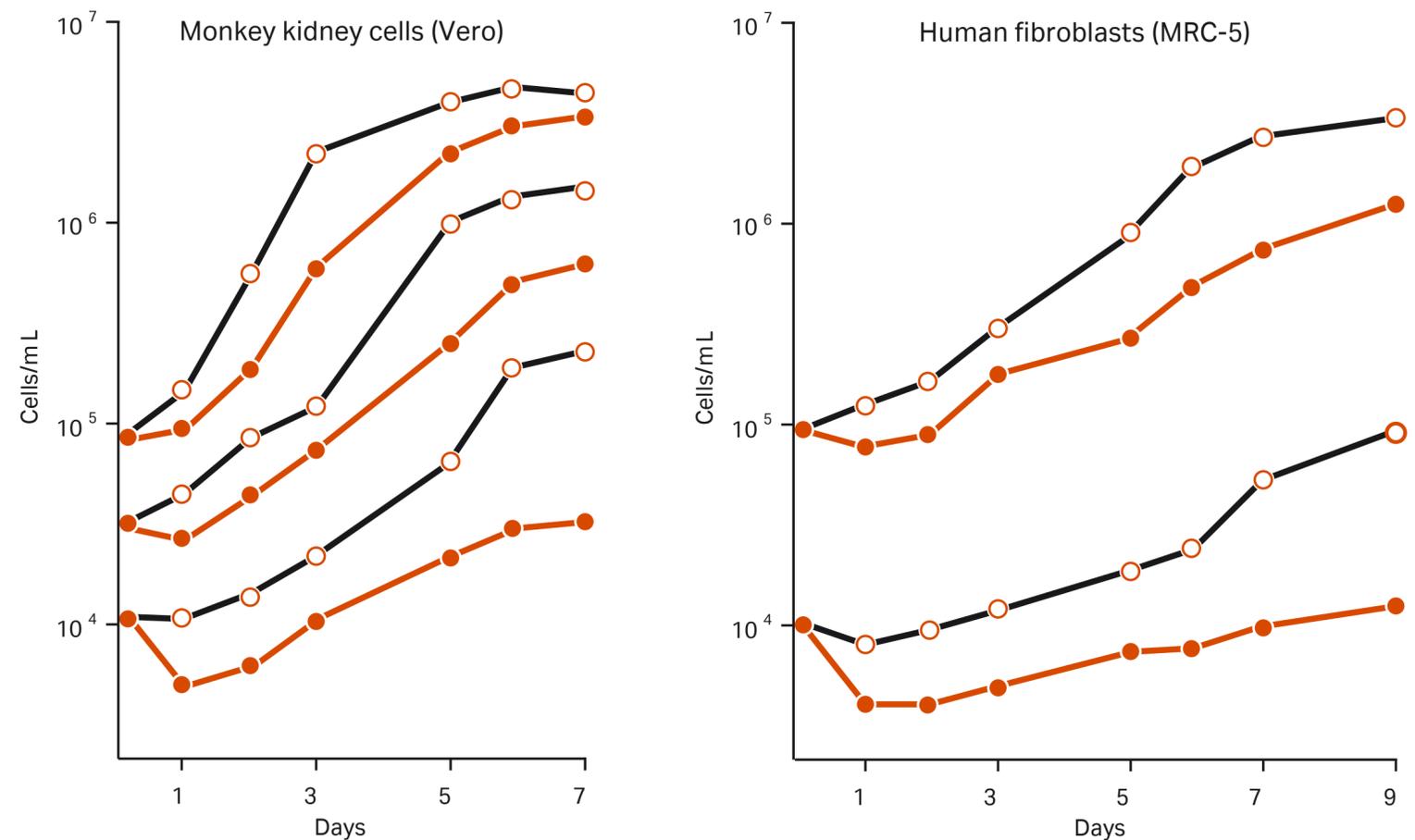
Fig 72. The effect of inoculation density and initial culture procedure on the growth of monkey kidney cells and human fibroblasts on Cytodex 1 microcarriers. Cultures were inoculated with cells and stirred immediately in the final volume (—●—, 60 rpm) or were cultured in a reduced volume during the attachment stage of culture (—○—). The modified initial culture procedure (—○—) involved stirring for 1 min (30 rpm) every 60 min in 1/3 of the final culture volume. After 4 h (Vero) or 6 h (MRC-5) the culture was diluted to the final volume and stirred (60 rpm). All cultures contained 3 mg Cytodex 1/mL final volume. (From Pharmacia Biotech AB, Uppsala, Sweden and Clark, J., Hirtenstein, M. Annals N.Y. Acad. Sci. **369**, 33 (1981) by kind permission of the authors and publisher.)

8.4.2.3 Concentration of microcarriers

The yield of cells from microcarrier culture is directly related to the surface area for growth and hence the concentration of microcarriers. In most situations, Cytodex microcarriers are used in stirred cultures at a concentration of 0.5–5.0 mg/mL final volume. With some types of cells (e.g., certain established cell lines), it is possible to achieve good growth at lower concentrations, but these are cells that can grow at low culture densities.

If an adequate supply of medium is provided (Section 8.4.3.2) and gas tension and pH controlled (Section 7.2, 7.3), it is possible to work with cultures containing more than 5 mg Cytodex/mL, and in some cases $5–10 \times 10^6$ cells/mL can be achieved. If consistent difficulties in maintaining culture conditions (pH and gas tension) or providing a sufficient supply of nutrients are encountered during the later stages of the culture cycle, consider decreasing the concentration of microcarriers. Within the range 0.5–5 mg Cytodex/mL final volume, the proportion of microcarriers bearing cells at the plateau stage of culture (and hence the yield) depends on the concentration of microcarriers (Fig 73). Under conditions where the absolute concentration of cells and microcarriers is low, the chance of a cell coming in contact with a microcarrier is small, and therefore a greater proportion of microcarriers remain devoid of cells at the plateau stage of culture.

If low concentrations of microcarriers must be used, increase the proportion of microcarriers bearing cells at the plateau stage of culture, and thereby the yield, with the modified initial culture procedure (Section 8.4.2.2). Provided a correct inoculation density is used (Section 8.4.2.4), a concentration of 3 mg Cytodex/mL final volume is usually optimal for general microcarrier culture and will result in the greatest proportion of microcarriers bearing cells.

The yield of cells/cm^2 from cultures containing lower concentrations of microcarriers (less than 2 mg/mL) depends on the ability of the cells to grow under less dense conditions. To obtain the maximum yield of cells/cm^2 from cultures containing higher concentrations (more than 4 mg Cytodex/mL), the culture medium may need to be replenished more often than when growing cells at a low concentration (Section 8.4.3.2). This is simply because a given volume of medium can only support the growth of a finite number of cells. As an approximate guide to expected cell yields, assume that the culture has a density of 10^5 cells/cm^2 at confluence. This corresponds to 6×10^5 cells/mg Cytodex 1 and 4.6×10^5 cells/mg Cytodex 3. The exact yield will depend on the characteristic saturation density of the cell type and on the supply of medium.

For Cytopore, the recommended carrier concentration is 2–3 g/L. Due to the high swelling factor of 40 mL/g, the concentration must be lower than with Cytodex. The maximum concentration is 5 g/L.

For Cytoline 2 in stirred tank applications, the concentration is between 100 and 200 mL/L of culture volume. Higher concentrations are only possible for very robust cells. For Cytoline in fluidized beds, carrier concentration depends only on the degree of expansion in the reactor. (Cell growth is independent of carrier load inside the fermenter.) The rate of microcarrier bed expansion depends on the attachment properties of the cells. The higher their adherence to the beads, the greater the expansion rate of the carrier bed inside the reactor and the lower the filling of the reactor. The maximum carrier load in a fluidized bed is half the working volume.
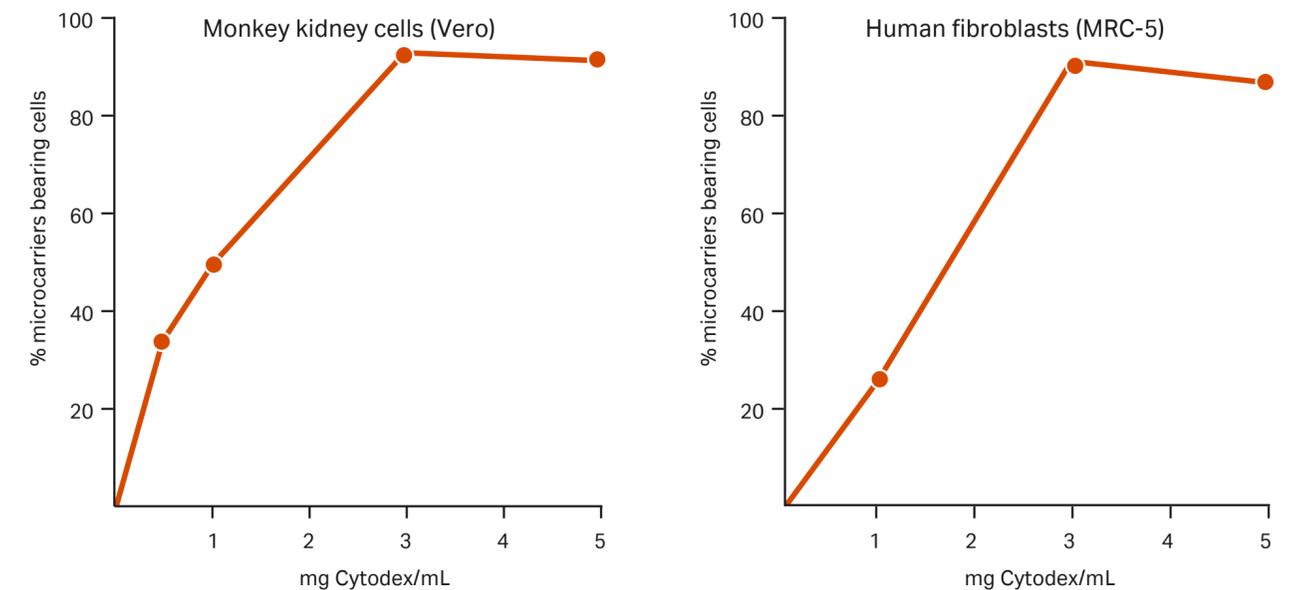
Fig 73. The effect of microcarrier concentration on the proportion of microcarriers bearing cells at the plateau stage of culture. Cultures were inoculated with 5 viable Vero cells/microcarrier or 10 viable MRC-5 cells/microcarrier and stirred immediately at 60 rpm. The proportion of microcarriers bearing cells was determined after 7 days (Vero) or 9 days (MRC-5). Cultures were maintained under conditions where supply of medium and control of pH were not limiting cell growth. (From Pharmacia Biotech AB, Uppsala, Sweden.)

8.4.2.4 Inoculation density

It is a general cell culture phenomenon that the survival and growth of cells depends to a large extent on the inoculation density and conditioning effects. These conditioning effects are dependent on the density of the culture; a low density leads to relatively poor growth. With respect to anchorage-dependent cells, one of the most critical parameters at inoculation is the number of cells/cm^2 of culture surface area. Cells with low plating efficiencies are particularly sensitive to culture under conditions of low density.

It is therefore important to take into account the large surface area provided by Cytodex microcarriers. Since the efficiency of cell attachment to Cytodex is similar to that observed in Petri dishes, the microcarrier cultures should be inoculated with approximately the same number of cells/cm^2 as when starting other types of monolayer cultures. The number of cells/cm^2 used to inoculate the culture will depend on the plating efficiency of the cells. When inoculating a culture, it is generally necessary to use more primary or normal diploid cells than established cells. Inoculation density affects both the proportion of microcarriers bearing cells at the plateau stage of culture and the yield from the culture (Fig 74). Figure 74 shows that approximately 10 human fibroblasts/microcarrier are required for maximum utilization of the microcarriers, whereas only 5 monkey kidney cells/microcarrier are required.

Horng and McLimans (45) reported that approximately 5 cells/microcarrier were required when inoculating cultures with anterior calf pituitary cells. If sufficient cells are not available, the modified initial culture procedure discussed in Section 8.4.2.2 can be used. Since this procedure results in a more efficient utilization of the inoculum, more microcarriers bear cells at the plateau stage of culture (Fig 74) and yields are increased (Fig 72). Further details of inoculation density can be found in Section 8.4.2.4.

On average, the inoculation density for Cytodex is around 2×10^5/mL of culture volume. For macroporous beads like Cytopore and Cytoline, the concentration should be around 2×10^6 per/mL of settled beads. A lower concentration might be deduced from plating efficiency or small-scale inoculation density tests.
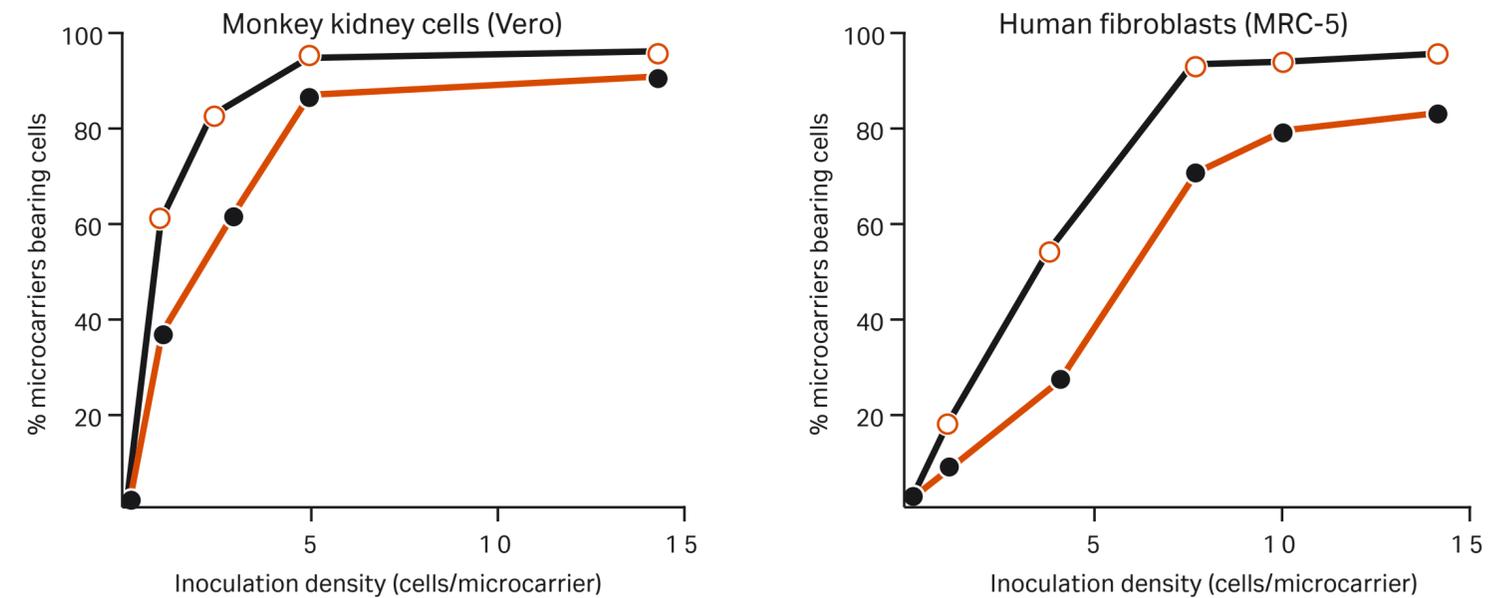
Fig 74. The effect of inoculation density on the proportion of microcarriers bearing cells at the plateau stage of culture. Culture contained 3 mg Cytodex 1/mL final volume and were either stirred continuously at 60 rpm (—●—) or were started with a reduced volume and intermittent stirring before the culture was diluted to the final volume and stirred at 60 rpm (—○—). Details of the modified culture procedure are given with Figure 76. The proportion of microcarriers bearing cells was determined after 7 days (Vero) or 9 days (MRC-5). (From Pharmacia Biotech AB, Uppsala, Sweden.)

8.4.2.5 Inoculum condition

The plating efficiency of cells depends on the stage of the culture cycle from which the inoculum is taken. Cells in exponential growth have a higher plating efficiency than cells from a resting population. The yield from microcarrier cultures can be increased 2–3 fold by inoculating with cells taken from exponentially growing cultures rather than confluent cultures (253). When possible, microcarrier cultures should always be inoculated with cells taken from actively dividing cultures at approximately 70% to 80% confluence. In this way, a greater percentage of cells in the inoculum attach to the microcarriers and contribute to the growth of the culture.

The inoculum should be evenly dispersed and preferably a single cell suspension. Avoid excessive centrifugation when concentrating the inoculum since this leads to aggregation of cells and reduced viability after resuspension. Centrifugation for 5 min at 200–300 g is normally sufficient. Suspending the inoculum in the medium to be used in the culture avoids diluting medium components. Also avoid exposing the inoculum to sudden changes in temperature, pH or osmolarity.

A general discussion on culture media can be found in Section 7.1, and replenishment is considered in Section 8.4.3.2 The nutritional requirements of cells are not necessarily the same throughout the culture cycle and for optimal results, it may be desirable to alter the formulation of the medium at some stage. This is usually necessary if the culture is to span a very wide range of densities e.g., from 5×10^4 cells/mL at inoculation to 3×10^6 cells/mL at confluence.

This section considers alterations to the medium during the initial stage of culture. The main difference between culturing cells as monolayers on microcarriers and on other culture surfaces is that microcarrier cultures span a wider range of cell densities for any single culture. Microcarrier cultures must often be started with a low number of cells/cm^2. The nutritional requirements of cells growing under conditions of low density are usually more stringent than for cells growing under high densities (155), and the cell plating efficiency can be improved by ensuring that the culture medium contains sufficient quantities of certain components.

The need to supplement the culture medium during the initial period depends on the medium, the inoculation density and the type of cell. For cells that tend to have high plating efficiencies (e.g., most established and transformed cell lines), additional supplementation of common culture media is usually not necessary. In contrast, many primary cells and normal diploid cell strains require additional supplementation if maximum yields are to be obtained. Such supplementation need only be included during the initial growth phase and is no longer required when the medium is replenished later in the culture cycle.

Table 7 shows that the growth of cells in cultures inoculated with small numbers of cells is better with a more "complete" medium with a large number of components (Medium 199) rather than with a more sparse medium (DME, BME). At high inoculation densities, plating efficiency is improved, and culture growth is greater in the medium with the highest concentration of amino acids and vitamins (DME).

8.4.2.6 Relationship between plating efficiency and culture procedure

Knowledge of the growth properties of a particular type of cell in general monolayer culture can be used to deduce near-optimal microcarrier culture procedures. This information often means that extensive preliminary experiments can be avoided and only minor adjustments to the culture procedure are needed to achieve the best results.

Comparative studies of the conditions required to culture a wide variety of cells in microcarrier culture reveal that plating efficiency is one of the most useful growth parameters to consider when developing a culture procedure (77, 78, 253). Plating efficiencies vary considerably between different cell types and are a measure of the cell's ability to survive a sub-culture step and contribute to the proliferation of the next culture. The plating efficiency of any particular type of cell is not a fixed value and can be influenced to a large extent by changing the culture procedure and/or conditions. If a cell possesses an intrinsically low plating efficiency, or if only small numbers of cells are available for inoculation, it is important to use culture procedures and conditions that enhance the plating efficiency.

Table 18 illustrates relationships between plating efficiency and initial culture variables. These relationships have been observed for a wide variety of cell types (77, 78, 253). For any type of cell, the essential elements of the initial culture procedure can be deduced from the plating efficiency. Primary cell suspensions normally have plating efficiencies of less than 10%, and the plating efficiency of normal diploid cell strains is usually between 10 and 30%. Most established cell lines have plating efficiencies greater than 30%.

Table 18. The relationship between the plating efficiency of a cell and those parameters known to be critical during the initial phase of a microcarrier culture

Parameter	Plating efficiency*		
	< 10%	10–30%	> 30%
Cells/microcarrier at inoculation (Section 8.4.2.4)	high (> 10)	intermediate (5–10)	low (< 5)
Initial culture volume (Section 8.4.2.2)	small (20–30% of final volume)	intermediate (30–60% of final volume)	large (100% of final volume)
Initial stirring speed** (Section 8.4.2.2)	static/intermittent	continuous (approx 10 rpm)	continuous (approx 40–60 rpm)
Additional medium supplements	required	advantageous	not required

* See text.
** Actual speed depends on design of stirrer and culture vessel.

In some cases, the culture procedure may need to be modified because of the specific growth properties of the cell type. For example, weak or slow attachment to culture surfaces and a rounded morphology suggest that stirring during the attachment phase should be very gentle and less frequent. In contrast, a tendency towards aggregation upon inoculation indicates that continuous but slow stirring will be required throughout the entire initial culture period.

Such peculiarities of growth can be checked by first performing a growth test on microcarriers in bacteriological Petri dish cultures every time a new type of cell is to be cultured (Section 6.1). The definition of plating efficiency used when developing a culture procedure need not be rigid. In the most accurate sense, plating efficiency is the proportion (%) of cells that can form colonies when plated at low density into a Petri dish (e.g., 200–500 cells/^{6} cm dish). In this case, the plating efficiency is measured as "cloning efficiency". Plating efficiency is also indicated by the routine "split" or sub-culturing ratio. A cell type that is sub-cultured routinely with a low split ratio (1:2) will usually require the initial microcarrier culture procedures for a cell with low plating efficiency (Table 18). Cells that can be sub-cultured with a high split ratio (e.g., 1:20) have high plating efficiency and modified initial culture procedures are not usually necessary.

Table 19 lists suitable inoculation densities for the microcarrier culture of some common established cell lines. Differences reflect differences in plating efficiency. The inoculation densities refer to cultures stirred continuously from the moment of inoculation. If fewer cells are available, intermittent stirring in a reduced initial culture volume should be considered (Section 8.4.2.2). If the plating efficiency or routine sub-culturing split ratio of a particular cell type is not known, the first cultures should contain 3 mg Cytodex/mL final volume and be inoculated with at least 10^5 cells/mL final culture volume. If necessary, modify the size of the inoculum for subsequent cultures.

Table 19. A guide to comparative inoculation densities for some common established cell lines

Inoculation density	Cell line
2×10^5/mL	Don, Detroit 532, NCTC 2544, RPMI 2650, SIRC
10^5/mL	Chan conjunctiva, BGM, BSC-1, CV-1, Y-1, Morris hepatoma, tumor virus transformed hamster cells (most), GL-V3, Pt-K-1
$8–9 \times 10^4$/mL	Chang liver, HeLa, MDCK, MDBK, HT 1080, LLC-MK$_2$, LLC-RK, J111, L-132, Vero, Neuro-2a, RK 13
$5–8 \times 10^4$/mL	CHO, HaK, Detroit 6, Detroit 98, Girardi heart, HEp2, KB, WISH, Chimp liver, 3T3, PK-15, C6, BHK 21, HTC, PyY, McCOY, L 929, A9, 3T6

These inoculation densities refer to cultures containing 3 mg Cytodex/mL and stirred from the moment of inoculation in the final culture volume. Lower inoculation densities can be used in combination with modified initial culture procedures (Section 8.4.2.2).

8.4.3 Maintaining a microcarrier culture

Once a microcarrier culture has been initiated, certain procedures and precautions are required to maintain cell proliferation and achieve maximum yield. If the culture is essentially non-proliferating, as in the case of some primary cultures (e.g., hepatocytes), conditions must enhance function and survival of the cells for as long as possible. Although the following comments will be restricted to proliferating cultures, exactly the same principles should be considered when maintaining non-proliferating cultures.

During the culture cycle, changes in the density of the cell population are usually greater than 10-fold. Such growth conditions the medium and thereby encourages cell growth until a saturation density is reached and density-dependent inhibition of proliferation occurs. (Note, however, that many established and transformed cell lines do not show such inhibition). At the same time, oxygen and medium components are utilized and toxic products of metabolism accumulate. These changes must be taken into account when maintaining a microcarrier culture. In addition, keep in mind when optimizing culture procedure that conditions optimal for cell growth at low density (e.g., gas tension, pH, etc.) are not necessarily optimal for later stages of culture (Section 7.2, 7.3). Always remember that the most important aspect of maintaining a microcarrier culture and obtaining the best results is to anticipate changes in the culture.

For example, from the first few cultures with a particular type of cell, it is possible to observe if or when pH changes occur, when oxygen supply or medium components are depleted and if aggregation of the microcarriers occurs. Once the stage at which these changes occur is known, corrective measures can be taken beforehand. It is often difficult to return to optimal conditions and obtain good results once large deviations have taken place and, in such cases, irreparable damage to the culture usually ensues.

8.4.3.1 Stirring speed

Although other culture systems can be used (Section 6.4.7), the most suitable method for maintaining microcarrier cultures (Cytodex, Cytopore) is stirred suspension. Stirring, a) ensures that the entire surface of the microcarrier is available for cell growth, b) creates a homogeneous culture environment, c) avoids aggregation of microcarriers by cell overgrowth, and, d) facilitates exchange of gases between the culture headspace and the medium, or oxygen transfer during sparging or via other oxygenators.

In principle, stirring speed should be just sufficient to keep all the microcarriers in suspension. After the initial culture period, during which there is normally intermittent stirring or a static attachment period (Section 8.4.2.2), the culture should be stirred continuously.

The rate of stirring influences greatly the growth and final yield of cells (Fig 75), an effect related to the integrated shear factor (Fig 76). Slower stirring speeds reduce shearing forces on cells attached to the microcarriers but, if the rate is too slow, growth is reduced (Fig 75). This effect is mainly due to inadequate gas diffusion, sedimentation and aggregation. If stirring is too fast, less strongly attached cells (mainly mitotic cells) dislodge from the microcarriers and there is no net increase in cell number. (Such a phenomenon can be used to advantage for harvesting mitotic cells (Section 4.5.5).)
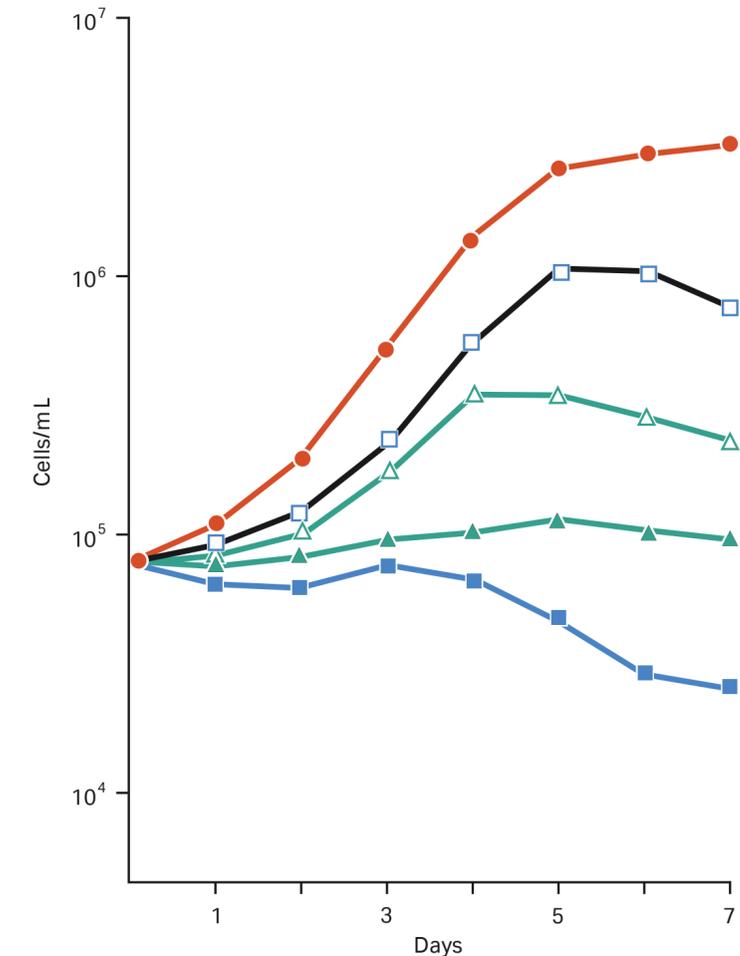
Fig 75. Effects of stirring speed on the growth of Vero cells on Cytodex 1 microcarriers (30 rpm (—▲—), 40 rpm (—□—), 60 rpm (—●—), 90 rpm (—△—), 120 rpm (—■—). All cultures were 250 mL and contained 3 mg Cytodex 1/mL. Culture vessels were traditional magnetic spinner vessels and the cultures were stirred from the moment of inoculation in the final culture volume. (Hirtenstein, M. and Clark, J. In "Tissue culture in medical research" eds Richards, R and Rajan, K., Pergamon Press, Oxford, pp 97 (1980), by kind permission of the authors and publisher.)

Excessive stirring speed causes a general loss of cells from the microcarriers and thereby poor cell yields (Fig 75, Fig 76).

Figure 76 illustrates that a population of cells growing on microcarriers is less sensitive to shear forces if an initial attachment phase is included in the culture procedure (Section 8.4.2.2). It is during the attachment phase of a stirred culture that the adverse effects of excessive shear forces are most noticeable. The stirring speed used during growth and plateau phases depends on the type of cell being cultured and on the design of the stirrer. Most primary cells and normal diploid strains attach firmly to culture surfaces and can withstand higher shear forces than more weakly attaching cells, e.g., many established or transformed cell lines. However, the most critical stage of the cell cycle regarding attachment is during mitosis, when most cell types do not differ greatly in attachment. Similar stirring speeds thus tend to be used for all cell types during the exponential phase of growth.

When cultures are to be maintained at high densities, a slight increase in stirring speed will improve gas exchange and can improve the supply of oxygen to the cells. However, if the culture is still dividing, an increase in stirring speed must be limited and should not result in detachment of mitotic cells. Some cells (mainly transformed cells and some fibroblast strains, e.g., chicken fibroblasts) tend to form aggregates during the later stages of culture (Fig 29 plate 5). In these cases, a slight increase in stirring speed will reduce this effect.

Increases in stirring speed must be considered carefully if there is any tendency for the cell monolayers to detach from the microcarriers. This detachment, which is discussed below, can be related to culture conditions. The other aspect of selecting correct stirring speed is the design of the stirrer and culture vessel, plus the volume of the culture. The optimal stirring speed when using traditional magnetic spinner vessels is usually 50–70 rpm (Fig 75), while speeds of 15–30 rpm are used with the modified spinner vessels or cultures stirred with bulb-shaped rods (Section 6.4.1). Higher speeds are often required when using the same design of stirrer with culture volumes larger than 500 mL. Gradually increasing stirring speed to these levels over a few days is recommended.

The stirring speed when working with large-scale culture volumes also depends on the design of the stirrer. Progressive increases from 50 to 100–150 rpm during the culture cycle are frequently used for cultures of 100 liters or more (4, 10, 254). With all types of stirring equipment, it is important to avoid sedimentation of the microcarriers, particularly during later stages of the culture. The accumulation of microcarriers that often occurs under the stirring axis in magnetic spinner vessels can be reduced by positioning the stirrer as close to the base as possible, while still leaving clearance for circulation of microcarriers. Such sedimentation does not occur with cultures stirred by a bulb-shaped rod, especially if contained in culture vessels with convex bases (Section 6.4, Fig 41).
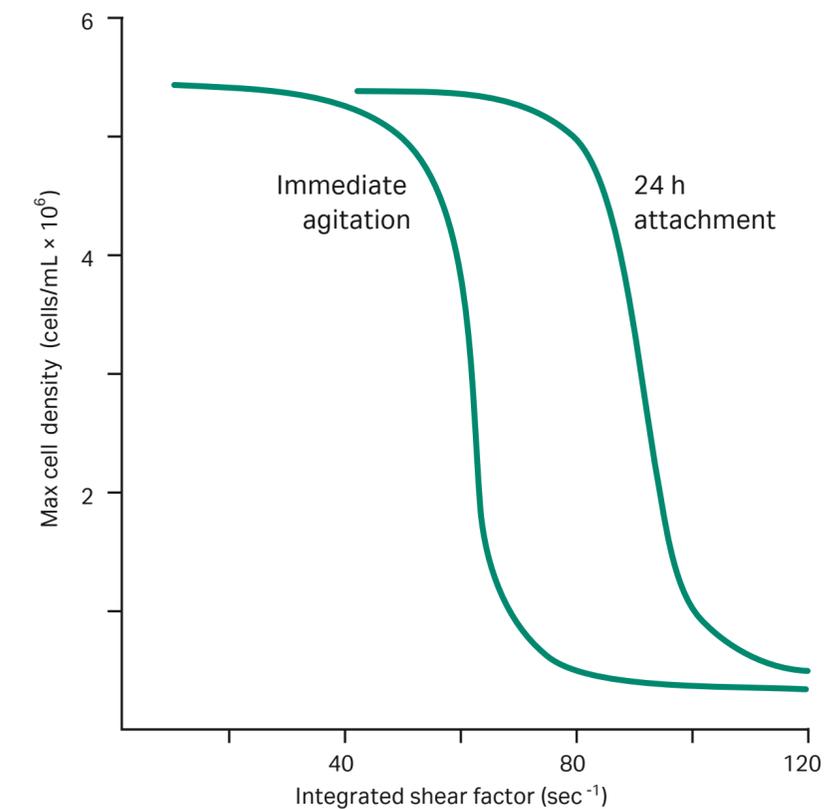

Fig 76. The effect of shear force on the productivity of microcarrier cultures of chicken embryo fibroblasts. (Sinskey, A.J., Fleischaker, R.J., Tyo, M.A. *et al*. Annals N.Y. Acad. Sci. 369, 47 (1981) by kind permission of the authors and publisher.)

When microcarrier cultures are used for production of viruses or cell products, the stirring speed should usually be reduced during the production phase. In most cases, a reduction to half that used during the growth phase is optimal. Excessive stirring or shear forces result in decreased yields of viruses or cell products (Fig 77).

8.4.3.2 Replenishment of culture medium

Careful replenishment of medium during the culture cycle is an important aspect of maintaining microcarrier cultures. There are three reasons for replenishing the medium: (A) replacing essential nutrients that are depleted by cell growth, (B) removing products of metabolism that inhibit growth or survival, and (C) assisting control pH. Careful planning of medium replenishment makes it possible to achieve maximum yields of cells for a given volume of medium.

The frequency and extent of medium replenishment depends on cell type, culture density, culture medium and gas tension. Rapidly dividing cells and cultures at high densities require more frequent replenishment than low density or slowly dividing cultures. Rapid cell division and high cell densities deplete medium components and decrease culture pH. At the same time, metabolites such as lactate, ammonia and even specific growth inhibitors accumulate (229, 255). The ideal replenishment scheme is the one that results in the smallest fluctuation of nutrient concentrations and pH during the culture cycle. For this reason, a continuous flow of medium is the preferred method for culture maintenance (232, 256). However, for small-scale cultures or experiments with cell densities up to $3–5 \times 10^6$ cells/mL batch, medium replenishment is more convenient.

The usual procedure is to start with replenishing 50% of the medium volume every 3 days. If necessary, a modified scheme can then be developed to get the best yield from the culture. For example, it is common practice to observe the culture every day and determine its density. When samples are taken, 10% to 20% of the medium volume can be replaced with fresh medium. To take advantage of conditioning effects, replenishment should not take place within the first 2 days of culture.

This replenishment scheme requires very little extra effort and usually results in higher yields. In this way, it is possible to avoid sudden changes in culture conditions, reduce fluctuations in nutrient concentration, reduce accumulation of toxic metabolites, and assist regulating gas tension and pH (sections 7.2, 7.3). When the cultures contain several million cells/mL and are in the exponential or plateau phase, more frequent replenishment will be required, or a modified culture medium used (see below). Fresh medium should have a pH and osmolality optimal for cell growth (Section 7.3, 7.4). Note: whenever added, fresh medium should have the same temperature as the culture.

Another approach to medium replenishment, especially during the later stages of exponential growth, is to feed the culture with a modified medium. During this stage, nutrient and growth factor requirements are not the same as at the beginning of the culture. Many medium components used when initiating the culture, e.g., non-essential amino acids, nucleosides, etc. can be omitted and greater economy achieved by reducing the concentration of the serum supplement (Section 7.1.1.9).
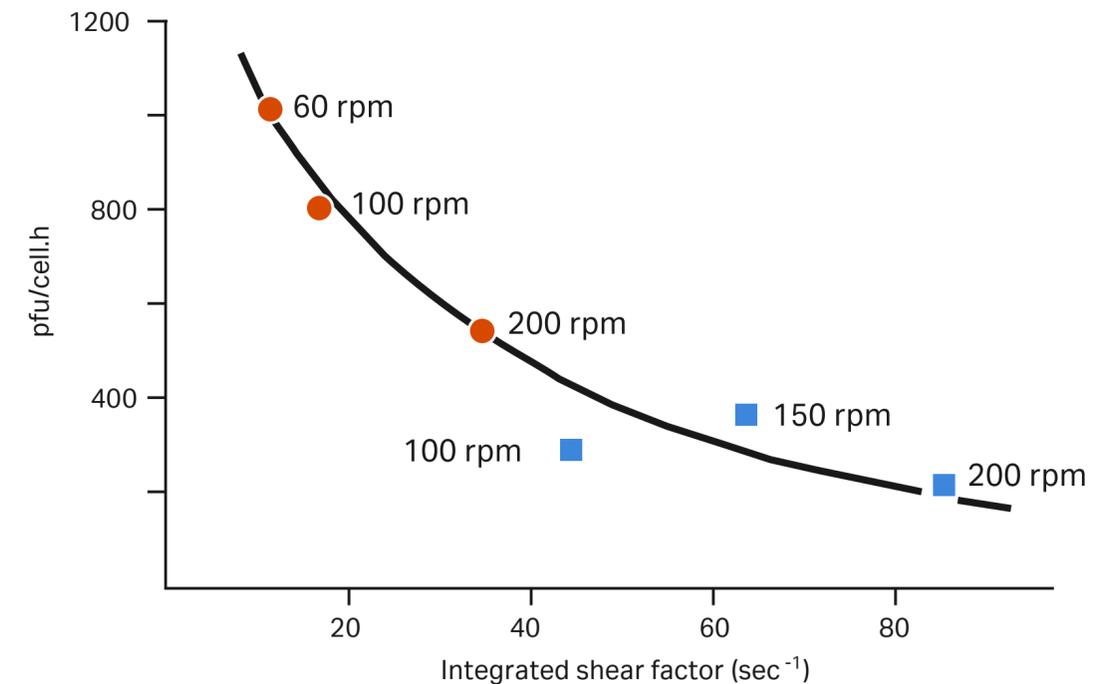
Fig 77. The effect of shear force on the production of Sindbis virus from microcarrier cultures of chicken embryo fibroblasts. Virus productivity was averaged for the final 8 h of infection. Cultures were contained in magnetic spinner vessels and stirring was with either a 4.4. cm (—■—) or 7.5 cm (—●—) impeller. (Sinskey, A.J., Fleischaker, R.J., Tyo, M.A. *et al*. Annals N.Y. Acad. Sci. **369**, 47 (1981), by kind permission of the authors and publisher.)

The type of culture medium can also be changed during the culture cycle. For example, cultures initiated with Medium 199 can be replenished by addition of DME. This change is beneficial because although Medium 199 is good for growth at low culture densities, DME is superior for high densities (Fig 79).

Persistent difficulties with controlling pH at high cell densities can be overcome by modifying the carbon source in the medium, increasing the oxygen tension slightly or by daily addition of glutamine in the presence of reduced concentrations of glucose (Section 7.3.3). If excessive aggregation of the culture occurs and cannot be controlled by adjusting stirring speed (Section 8.4.3.1), the calcium and magnesium concentration in the medium can be reduced. A simple method for reducing these ions is to use mixtures of media that include suspension culture versions (e.g., Spinner MEM). A 50:50 mixture of this medium and the usual culture medium normally overcomes difficulties with aggregation, without affecting cell growth.

When nutrient depletion rather than accumulation of toxic metabolites or decrease in pH is the growth-limiting factor in a culture, a different approach can be adopted. Growth limitation is caused by depletion of only certain medium components. Provided other factors are not limiting, replenishing these components leads to continued cell growth. Figure 78 illustrates that a stock solution containing these components can be added to microcarrier cultures of chicken fibroblasts with the result that cell yields can be as good as those when complete medium is used for replenishment. This method gives better economy, but requires good control of pH and a clear definition of limiting nutrients.

Fig 79. The effect of various culture media on the growth of monkey kidney cells (Vero) on Cytodex microcarriers. (DME, ●; BME, ○, Medium 199, △). All media were supplemented with 10% (v/v) fetal calf serum. Cultures contained 5 mg Cytodex 1/mL and were stirred at 60 rpm for the culture period. In all experiments 50% of the medium was changed on day 3. (Clark, J.M., Hirtenstein, M.D., Annals N.Y. Acad. Sci. **369**, 33 (1981) by kind permission of the authors and publisher.)

Fig 78. The depletion of medium components during growth of secondary chicken fibroblasts in microcarrier culture. Cells were cultured in DME supplemented with 15 mM HEPES. 5% (v/v) calf serum, 1% (v/v) chicken serum and 1% (w/v) tryptose phosphate broth and containing Cytodex 1 microcarriers (5 mg/mL). After 3 days the culture medium was replaced by fresh medium (—○—) or was removed and supplemented with cystine (30 µg/mL), glutamine (0.3 mg/mL), inositol (2 µg/mL), glucose (2 mg/mL), choline HCl (1 µg/mL) and 1% (v/v) calf serum, 1% (v/v) chicken serum. The medium was well mixed and returned to the culture (—▲—). Control cultures were not refed (—●—). Replenishment of all the medium was necessary after 7 days if the microcarriers were to be kept confluent. (Clark, J., Hirtenstein, M. Annals N.Y. Acad. Sci. **369**, 33 (1981) by kind permission of the authors and publisher.)

During maintenance of large-scale cultures, nutrients such as amino acids may be utilized differently in different culture systems. Figure 80 compares the utilization of serine and arginine in a continuous stirred tank and a fluidized bed (257). For serine, the situation is constant for both systems. For arginine, the situation is critical at certain glucose levels. Arginine thus has to be added to the culture medium during replenishment.

All these aspects of medium replenishment apply to large-scale microcarrier culture. An advantage with large-scale cultures is that parameters such as pH and gas tension are better controlled than with small-scale closed culture systems. It is thus easier to optimize replenishment schemes.

Closed culture systems are frequently used for microcarrier culture at laboratory scale. With such systems, the vessels are sealed and the supply of gas is only renewed when the culture is opened for sampling or replenishment of the medium. One important aspect to consider when working with such culture systems is the ratio of the culture volume to the total internal volume of the vessel. Figure 63 illustrates that the extent to which a spinner vessel is filled with culture influences greatly the maximum yield of cells/mL from the culture. Therefore, for reproducible results, closed culture vessels should always be filled to the same extent (and not more than half full). The reduction in yield in closed culture vessels that are more than half full is probably due to a decreased supply of oxygen and reduced headspace volume for buffering the usual CO_2-bicarbonate system. This phenomenon is not encountered with open culture systems having a continuous gas supply. More information on culture media, gas and pH control can be found in sections 7.1, 7.2 and 7.3, respectively.
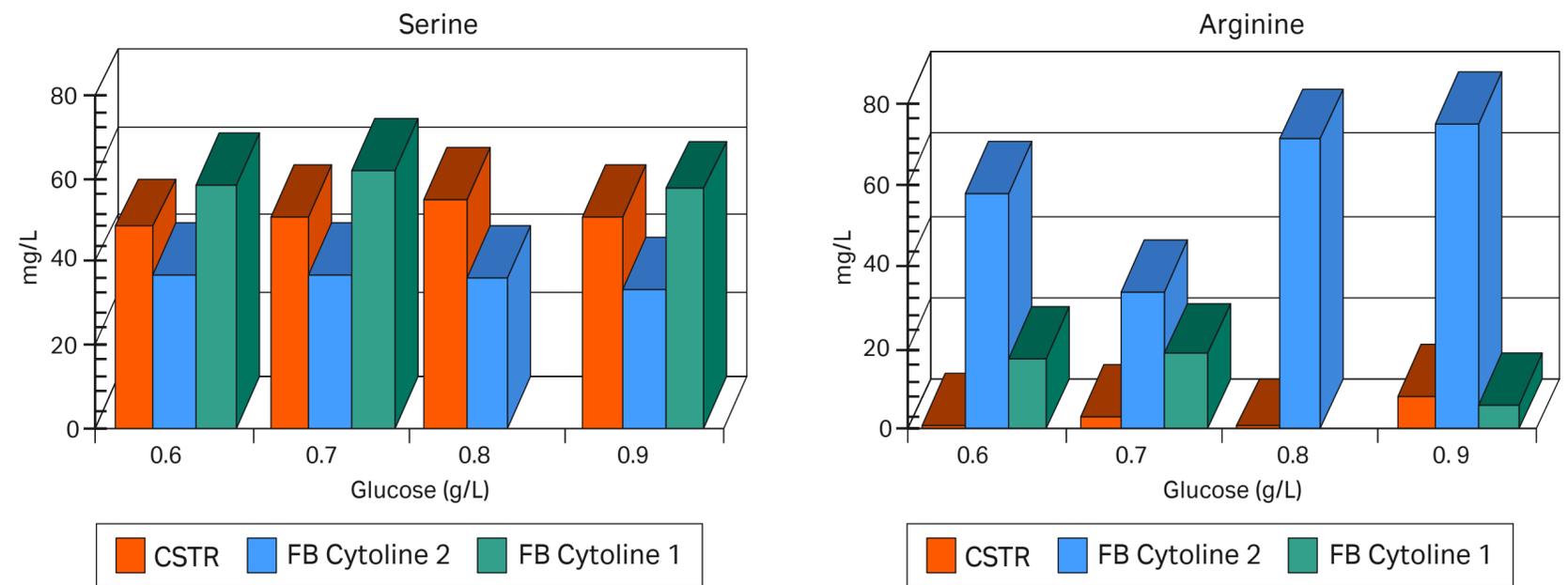
Fig 80. Different amino acid consumption with the same cell line (human hybridoma) and media in different reactor systems. Reactors compared: Stirred tank reactor in continuous operation (CSTR), Cytopilot using Cytoline 1 (FB Cytoline 1) and Cytopilot using Cytoline 2 (FB Cytoline 2) microcarriers.

8.4.3.3 Maintaining cultures at confluence

Production from microcarrier cultures sometimes requires that monolayers of cells be maintained at confluence on the microcarriers for several days or even weeks. Fibroblast interferon, certain viruses and urokinase are examples of products where viable and functional monolayers must be kept for extended periods.

Mered *et al.* (3) describe the maintenance of monolayers of chicken embryo fibroblasts and Vero cells for periods of 1 month or more without detachment or loss of viability. Dog kidney cells can be maintained on the microcarriers for periods of up to 38 days during rabies vaccine production (91). Similar procedures are used to maintain confluence on Cytodex as are used in other monolayer culture systems. Cells that are contact-inhibited for proliferation (e.g., primary cells, diploid cell strains and several established cell lines) require stable culture conditions that promote viability and function of the quiescent population of cells. Such cells often form monolayers that become only weakly attached to culture surfaces when saturation density is achieved.

A typical example is the tendency for highly confluent monolayers of chicken embryo fibroblasts to detach from Petri dishes, roller bottles or microcarriers. This phenomenon can be prevented by careful control of pH, reduced supply of serum supplements, and consistent supply of fresh medium.

For cell types that do not show contact inhibition of proliferation, a reduction in the formation of multilayers of cells on the microcarriers must also be accompanied by careful control of pH. Multilayers of cells growing on microcarriers (Fig 29 plate 5) are very sensitive to changes in culture conditions and may detach with only small fluctuations in pH or nutrient supply.

The following general points should always be considered when maintaining microcarrier cultures at confluence. (They are not as critical for macroporous microcarriers as for surface microcarriers because the cell multilayers are protected inside the pores.)

- **pH.** It is most important to maintain optimum culture conditions, especially pH. Once a drift in pH occurs (usually a decrease) cells tend to detach, even after pH has been returned to the optimal level. The most common cause of a decrease in pH at the later stage of the culture cycle is accumulation of lactate (Section 7.3.3).

- **Osmolarity.** When pH is being controlled by addition of acid/base or buffers, it is important to avoid changes in osmolarity (Section 7.4).

- **Serum concentration.** The most common way of maintaining cultures at confluence is to reduce the concentration of the serum supplement. A reduction from the usual 5% to 10% (v/v) supplement to 2% to 5% (v/v) is required for cells that are contact-inhibited for proliferation. For cells that continue to divide after confluence, consider lower concentrations of serum (down to 0.5% v/v).

- **Medium replenishment.** The concentration of nutrients should be kept as constant as possible, and toxic products of metabolism should not be allowed to accumulate. Thus, a consistent supply of medium should be ensured, for which daily replacement of 10% to 20% of the medium usually gives the best results. For many cell types, depletion of medium components is not as rapid at confluence as during earlier stages, and the main function of medium replenishment is to control pH. Temperature shocks should always be avoided and all solutions pre-warmed to the culture temperature. Daily addition of low concentrations of glutamine instead of occasional addition of medium with high concentrations of glutamine avoids unnecessary accumulation of ammonia, a toxic product of glutamine decomposition (177, 258). Hence, glutamine-free medium and daily addition of approximately 0.1–0.2 mM glutamine assists in maintaining viable monolayers by providing a more constant level of this essential amino acid without excessive accumulation of ammonia. If cultures are not contact inhibited for proliferation and continue to divide after confluence, higher levels of glutamine in the presence of low concentrations of glucose will assist in maintaining pH (Section 7.3).

- **Antibiotics.** When possible, decrease the concentration of antibiotics in the culture medium for long-term maintenance of confluent cultures.

Control of stirring speed and gas tension is discussed in sections 8.4.3.1 and 7.2 respectively.

8.5 Cell quantification

Cell numbers can either be determined directly or indirectly.

(A) Direct methods include cell enumeration by counting whole cells (attached cells trypsinized) or crystal violet stained nuclei (259) or by determining total cell mass via protein or dry weight measurements. Cell viability is more difficult to quantify, but is often done via trypan blue (0.4%) or erythrosine B (0.4%) staining. An alternative is the MTT colorimetric method (67). Some cell culture systems cannot be sampled, and therefore numbers cannot be determined directly nor morphology studied via hematoxylin staining/fixation.

(B) Indirect methods measure metabolic activities such as glucose or oxygen consumption, lactic or pyruvic acid production, CO_2 production, and increase in product concentration. These are quite useful during logarithmic growth but can be misleading later. Another possibility is to measure enzyme concentrations in the culture. One example is lactate dehydrogenase (LDH), which is not so dependent on the different phases of growth. A relatively new technique is to measure capacitance (Aber instruments). The advantage of this technology is that it measures cell mass even within macroporous carriers, so it continuously monitors cell growth within the bioreactor.

All these cell counts give a good picture of cell growth if you always use the same detection method. It is nearly impossible to compare different culture runs (performed with different cell lines) measured with different cell counting methods. This is demonstrated in the article of Capiaumont *et al*. (260), where they found up to a factor of eight difference between the various cell counting methods (Table 20).

Table 20. Cell concentration determination techniques

Parameters	Cell count at the end of culture $(10^6/mL)$
Glucose	2.5
Lactate	4
Alkaline phosphatase	1.14
β-Glucuronidase	0.9
Alanine	0.9
Mean glucose/lactate	3.2
Counted nuclei	3

8.5.1 Direct observation by microscopy

Examining cells by microscopy is a vital part of microcarrier culture technique. For routine observation, the growth and condition of the cells can be assessed simply with phase contrast optics. A small sample of evenly suspended culture is placed on a microscope slide and a coverslip is gently lowered over the sample. To avoid crushing the microcarriers, the coverslip should come to rest slightly above the slide. This can be accomplished by placing small pieces of broken coverslip between the slide and the coverslip. Haloes occasionally form with phase optics, but can be avoided by increasing the refractive index of the medium, for example by the addition of serum or Ficoll 400 (as an isotonic 30% (w/v) stock). If permanent preparations are required, the sample must be fixed (Section 8.5.4) and then stained (Section 8.5.5). Quantifying cell growth can be achieved by counting cells attached to individual microcarriers, but this is generally too time-consuming to be useful for routine purposes. More efficient and rapid methods for determining cell number are described below.

8.5.2 Counting cells released after trypsinization

A 1 mL sample of evenly suspended culture is placed in a test tube and after the microcarriers have settled, the supernatant is removed and the microcarriers briefly washed in 2 mL Ca^{2+}, Mg^{2+}-free PBS containing 0.02% (w/v) EDTA, pH 7.6. When the microcarriers have settled, this solution is decanted and replaced by 1 mL of a 1:1 mixture of 0.25% (w/v) trypsin in Ca^{2+}, Mg^{2+}-free PBS and EDTA (0.02%, w/v) in Ca^{2+}, Mg^{2+}-free PBS. The pH of this mixture should be 7.6. The tube is incubated at 37°C for 15 min with occasional agitation. The microcarriers are allowed to settle and the supernatant is transferred to another test tube. The microcarriers are washed with 2 mL culture medium containing serum (5% to 10%, v/v) and the supernatant is pooled with the first supernatant. The cell suspension is centrifuged (300 gav, 5 min, 4°C), the supernatant discarded and the pellet resuspended in 2 mL Ca^{2+}, Mg^{2+}-free PBS containing 0.05% (w/v) trypan blue. The concentration of cells in the suspension can be counted in a hemocytometer or electronic counter. The concentration of the cells in the culture can be expressed per mL or per cm^2 of microcarrier surface area. Including trypan blue in the re-suspension solution allows cell viability to be estimated at the same time. A similar method can be employed when using collagenase in combination with Cytodex 3 microcarriers (Section 8.6.1.2).

8.5.3 Counting released nuclei

A simpler way of monitoring cell growth is to count released nuclei as described by Sanford *et al.* (261). In this method, modified by van Wezel *et al.* (70), cells growing on the microcarriers are incubated in a hypotonic solution, and nuclei released by lysis are stained by a dye in this solution. A 1 mL sample of evenly suspended culture is centrifuged (22 gav, 5 min) and the supernatant discarded. The pelted microcarriers are resuspended in 1 mL 0.1 M citric acid containing 0.1% (w/v) crystal violet. The contents of the tube are mixed well (e.g., with a "Whirlimixer" or by several traverses over a corrugated surface) and then incubated for 1 hr at 37°C. Avoid evaporating the tube contents by using either a humidified incubator or by sealing the tube with plastic film. After incubation, the contents of the tube are mixed as above and the released stained nuclei are counted with a hemocytometer. Microcarriers in the sample do not interfere with the counting, and samples can be stored for up to 1 week at 4°C. This method of determining the number of cells in the culture is most accurate when cultures are evenly suspended and when culture conditions have avoided aggregation of microcarriers and cells (Section 10).

8.5.4 Fixing cells

When fixation and staining are necessary, for example for preservation of samples, cytochemistry, electron microscopy etc., any of the usual cell culture fixation and staining procedures can be used with Cytodex microcarriers (Fig 29, plates 1–9).

Methanol and ethanol are the most common fixatives. When using such alcohols, maximum preservation of cell morphology can be achieved by first rinsing the cells and microcarriers with warm PBS and then pre-fixing in 50% (v/v) alcohol in PBS. After 10 min, replace the 50% alcohol by two 10 min changes of cool 70% (v/v) alcohol in PBS. Finally, replace this fixative by 70% (v/v) alcohol in water. Alternatively, use a modified Carnoy's fixative (3 parts methanol, 1 part glacial acetic acid and containing 2% (v/v) chloroform) after the cells and microcarriers have been rinsed in PBS.

Better preservation of morphology will be achieved when aldehyde fixatives are used. Use either 10% (v/v) formaldehyde in PBS or 2% to 5% (v/v) glutaraldehyde in PBS and fix the material overnight at 4°C. Glutaraldehyde fixation results in the best preservation of morphology, and the fixed material can be used for electron microscopy studies (Fig 29, plate 1).

Further processing of the fixed cells attached to the microcarriers depends on the purpose of the study. For example, using standard procedures, the material can be dehydrated in a graded series of alcohol solutions, cleared in xylene and embedded in paraffin (45). For electron microscopy, cells growing on the microcarriers can be fixed, embedded, sectioned and stained by the usual procedures.

Dehydration of the microcarriers in acetone instead of alcohol avoids the use of propylene oxide, which has been reported to alter the surface of microcarriers (98). When sections through cells attached to a solid surface are required, cells growing on Cytodex are easier and more convenient to process than cells growing on the surface of Petri dishes or coverslips. When processing microcarriers with cells attached for microscopy, remember that the time taken for each step should allow for penetration of the microcarrier matrix by the solute or embedding agent, which doubles the usual process times for embedding. Examples of transmission and scanning electron microscopy of cells attached to Cytodex can be seen in Figures 4 and 8 and Figure 29 plates 1, 4, 8.

Pawlowski *et al*. (114) used the following procedures for preparing the cells in plate 1. Cytodex with cells attached was allowed to settle onto coverslips coated with gelatin (1%) and fixed in half-strength Karnovsky's fixative (4% glutaraldehyde, 1% paraformaldehyde, 0.1 M sodium cacodylate buffer, pH 7.8 and 12 mg $CaCl_2$/100 mL) for 30 min. The microcarriers were then washed briefly in 0.1 M cacodylate buffer (pH 7.8) and post-fixed in 1% osmium tetroxide for 1 hour. Samples were dehydrated in a graded ethanol series and critical point dried with carbon dioxide. The coverslips were "spatter-coated" (gold coating device) with gold for 1.5 min at 25 mA and 1.5 kV (114; R. Przybylski, pers. comm., 262).

8.5.5 Staining cells

The most suitable routine procedures for staining cells growing on microcarriers use either Geimsa stain or Harris' hematoxylin. The latter stain can be used when better nuclear detail is required.

8.5.5.1 Staining with Giemsa stain

Microcarriers with cells attached are rinsed in a small volume of warm PBS. Fixation in 50% (v/v) methanol in PBS for 10 min is followed by dehydration in a cool graded methanol series (70%, 90%, 95% v/v solutions in PBS) to absolute methanol, with about 5 min at each concentration. The material is stained for 5 min in May-Grünwald's stain (alternatively Jenner's or Wright's stain) and for a further 10 min in dilute Giemsa (1:10 volumes in distilled water). A brief rinse in water will educe staining to the required intensity.

8.5.5.2 Staining with hematoxylin

Cytodex with cells attached is rinsed in a small volume of warm PBS. After fixation in 50% (v/v) methanol PBS for 10 min, the microcarriers are fixed for a further 10 min in cool 70% (v/v) methanol in PBS. The fixative is removed and 10 mL distilled water containing 2–3 drops of hematoxylin are added. The material is left overnight at room temperature and then rinsed in tap water for 20 min. If desired, the cells can be counter-stained in an aqueous solution of eosin-Y for 30 sec and, for permanent storage, the material can be dehydrated in a series of alcohol solutions (50%, 70%, 90%, 95% v/v solutions in distilled water) and two changes of absolute alcohol. The material can then be cleared in xylene and mounted.

Many other more specific staining procedures are possible. Note, however, that because of the nature of the carbohydrate matrix of the microcarriers, it may not be possible to use some carbohydrate-specific stains. Some protein-specific stains will also stain the collagen layer on Cytodex 3. When staining dense monolayers of cells, it may be necessary to use slightly longer times for rinsing. This will ensure that free stain is washed from the microcarrier matrix. When mounting the microcarriers for examination, ensure that they are not crushed by the glass coverslip. This can be avoided by raising the coverslip above the surface of the slide with small fragments of broken coverslips placed on the slide. Figure 29, plate 3 shows an example of immunostained cells attached to Cytodex microcarriers.

8.6 Scale-up

Scale-up generally means a lengthy development period to ensure that all parameters are under firm control. Try to work, therefore, with a limited number of cell lines. Process simulation is also an essential part of scale-up. This determines if cells cope with scale-up without alterations and with maintained productivity. The viability and productivity of the cells has to extend beyond the planned time of the production process.

An essential part of scale-up is the cellular multiplication at each scale-up step. If it is possible to inoculate at a low cell density, i.e., 10^4/mL and the final yield is 10^6/mL, the multiplication factor is 100-fold. The larger the multiplication factor at each step of scale-up to final production volume, the fewer steps are needed to achieve it. Maximizing the multiplication factor minimizes the number of steps/operations needed! It also affects the investments required. If the above applies, it is possible to inoculate a 100 liter fermenter from a 1 liter spinner. In the case of colonization (see Section 8.6.2.2), a 1 liter spinner could inoculate a 1000 liter fermenter.

Scaling-up by volume normally involves moving from glass to stainless steel vessels, from mobile to static systems, and from autoclavable to *in situ* sterilization. Additional equipment is also needed at larger scale; seed vessels, medium hold tanks and sophisticated control systems, for example.

Scaling-up microcarrier cultures (Fig 81) can be done by increasing the size of the vessel or by increasing the microcarrier concentration. Production units up to 6000 liters (Fig 2) have been achieved by increasing size. Factors that influence this strategy include reactor configuration and the power supplied by stirring. The height to diameter ratio is one important factor. For surface aeration, the surface area/height ratio should be 1:1. Variables that affect impeller function include shape, ratio of impeller to vessel diameter and impeller tip speed. Larger impellers at lower speeds generate less shear force. Marine impellers have been found to be more effective for cells (Fig 81). Pneumatic energy supplied via air bubbles or hydraulic energy in perfusion can both be scaled up without increasing power input.

Desirable features of a stirred microcarrier reactor include no baffles, curved bottom for better mixing, double jacket for heating and cooling, top-driven stirrer, and a smooth surface finish (electropolished). Scale up by increasing the microcarrier concentration requires perfusion, which makes it necessary to have a separation device to keep the carriers in the reactor. A settling zone or a spin filter are both suitable for this task (see Section 6.6).

The limiting factor for higher cell concentrations is usually oxygen supply. It is difficult to use direct sparging with normal microcarriers in stirred tanks as the carriers may accumulate and float in the foam created. This can, however, be achieved by using large bubbles or by sparging inside a filter compartment. Other alternatives that increase oxygen supply include increasing surface aeration, perfusion via an external loop and oxygenation devices/vessels (263).

Fig 81A. 500 L stirred tank for microcarrier applications.

Fig 81B. 2000 L tank for microcarrier applications.
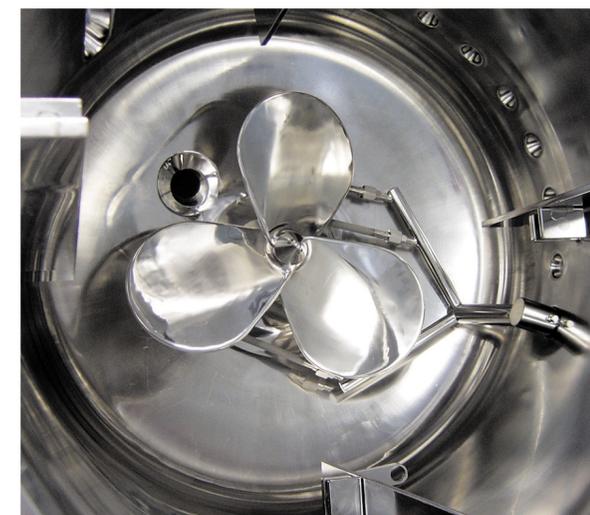
Fig 81C. Marine impeller of large- scale stirred tank. Courtesy of Werthenstein Chemie Ag, Switzerland.

It is easier to increase oxygen supply via sparging in packed or fluidized beds as the cells are protected inside the macroporous carriers. Scale-up of fluidized beds is linear, i.e., the diameter to height ratio is the same, which keeps the fluidization velocity constant. As oxygen is supplied via direct sparging with microbubbles (fluidized bed with internal loop), there is a continuous gas-to-liquid transfer throughout the bed. This makes the technology really scalable (Fig 82). Airlifts have been scaled up in this way to 10 000 liter scale!

8.6.1 Harvesting cells

Removing cells from microcarriers is usually required when sub-culturing and scaling-up, and also when large numbers of cells are required for biochemical analyses. However, it is important to note that for many biochemical studies, e.g., isotope incorporation studies, it may not be necessary to remove the cells from Cytodex. The only precaution required is that the microcarriers are well washed with buffer and precipitation agent (usually 5% to 10% trichloroacetic acid) so that all non-incorporated isotope is washed from the matrix.

Various methods can be used to remove cells from Cytodex, and it is important to choose procedures that minimize damage to cells. In most cases, common cell culture methods are used with only minor modifications to standard techniques. The type of harvesting procedure can usually be deduced from that normally used to harvest a particular type of cell from other types of monolayer culture. Enzymatic methods for removing cells from microcarriers (Section 8.6.1.2) are most commonly employed. Since cells are less strongly attached at alkaline pH, it is useful to use solutions with pH 7.6 to ensure that all acidic culture medium is removed prior to harvesting. Unless otherwise stated, all solutions should be pre-warmed to 37°C.

The aim of this step should be to have an inoculum consisting of a single cell suspension of highly viable cells that were in logarithmic growth prior to harvesting. To achieve this, it is necessary to develop and follow a strict harvesting protocol to apply after the carriers have sedimented and the supernatant been decanted.

The main task is to break the cell-surface and cell-cell interactions (the cells have to be harvested prior to confluence) and to round up the cells. As cell binding is dependent on divalent ions, the carriers have to be washed with citric acid or EDTA (0.2% w/v) containing buffers (PBS) to help detach the cells. If the media contains serum, alpha-1-anti-trypsin, a trypsin-blocking protein abundant in serum, has also to be washed out. Microporous carriers require more extensive washing compared with solid carriers; normally 2 washings (optimize depending on the cell/microcarrier/medium) with a volume of washing solution equal to the sedimented microcarrier volume.

Fig 82A. Large-scale Cytopilot reactor (100 L). Courtesy of Vogelbusch GmbH, Vienna, Austria.
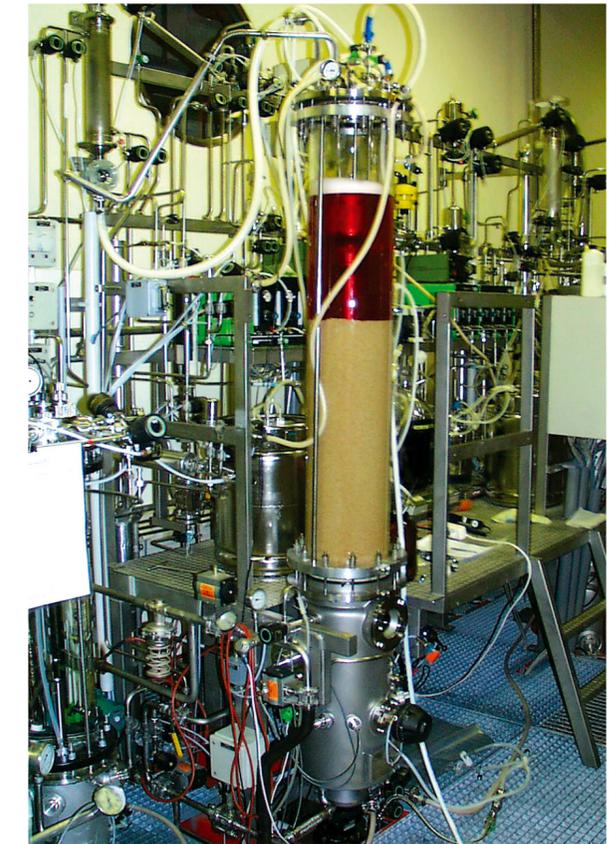
Fig 82B. Large-scale production reactor at Institute of Applied Microbiology, Vienna (Polymun Scientific, GmbH).

As trypsin has a pH optimum close to pH 8, it may be necessary to increase the buffer capacity of the PBS used for harvesting to maintain a pH of 7.4 throughout the procedure. Note that the substrate concentration for trypsin increases drastically for sedimented beads compared with ordinary flask cultures as the cell density becomes very high! Try to maintain the same ratio of units of trypsin/number of cells. Normally, this means increasing trypsin concentration 10-fold (i.e., 0.25% w/v, but this may depend on the supplier) compared with harvesting flasks. To speed up harvesting, pre-warm both the washing solution and the trypsin solution to 37°C. As trypsin activity is dependent on Ca^{2+} ions, it is advantageous to separate the EDTA washes and the final trypsinization. Screen different suppliers for a suitable trypsin for a particular cell line. Do not use "crystalline" trypsin. Normally, it is better to use a trypsin contaminated with other proteases. This is usually more effective!

Some shear force may have to be applied to quickly detach the cells. In spinners, stirring speed can be increased during this step or flasks shaken. At larger scale, the bioreactor can be emptied into a special harvesting reactor developed by Van Wezel (available from B. Braun) (Section 8.6.1). The trypsin has to be inactivated before the cells can be used as inoculate. Add either serum to the harvest or aprotinin, soybean trypsin inhibitor, if serum-free cultures are required. If serum is used, add it at the same time as the cells.

8.6.1.1 Chelating agents

Chelating agents such as EDTA can be used to remove certain epithelial and transformed cells from Cytodex. After removing and discarding the medium, wash the microcarriers twice in Ca^{2+}, Mg^{2+}-free PBS containing 0.05% (w/v) EDTA (100 mL/g Cytodex). The microcarriers are then incubated at 37°C with fresh Ca^{2+}, Mg^{2+}-free PBS containing 0.05% (w/v) EDTA (approx. 50 mL/g Cytodex). Stir the mixture continuously in the culture vessel at approximately 60 rpm for at least 10 min. Stirring speed may need to be increased or aspiration with a pipette may be required for some types of cells. When the cells have detached from the microcarriers, neutralize the EDTA by adding culture medium (100 mL/g Cytodex). The detached cells can be separated from the microcarriers as described in Section 8.6.1.9. In general, chelating agents alone are not sufficient to remove most cell types and are therefore usually used in combination with proteolytic enzymes. Long periods of exposure to EDTA may be harmful to some fibroblast strains, and EDTA alone rarely removes this cell type.

8.6.1.2 Proteolytic enzymes

Enzymes are normally used for routine harvesting of a wide variety of cells from Cytodex microcarriers. In general trypsin, VMF Trypsin, Pronase™ (Merck KGaA) or Dispase™ (GODO SHUSEI Co., Ltd.) are used with all microcarriers and collagenase is used in combination with Cytodex 3. Trypsin is the most commonly used general protease, although Pronase has advantages for harvesting cells from primary cultures, and Dispase can be used for cells that are sensitive to trypsin.

Trypsin: Stirring is stopped and the microcarriers allowed to settle. The medium is drained from the culture and the microcarriers are washed for 5 min in Ca^{2+}, Mg^{2+}-free PBS containing 0.02% (w/v) EDTA, pH 7.6. The amount of EDTA-PBS solution should be 50–100 mL/g Cytodex. The EDTA-PBS is removed and replaced by trypsin-EDTA and incubated at 37°C with occasional agitation. After 15 min, the action of the trypsin is stopped by addition of culture medium containing 10% (v/v) serum (20–30 mL medium/g Cytodex). An alternative method for inactivating the trypsin is to add soybean trypsin inhibitor (0.5 mg/mL). Chicken serum does not contain trypsin inhibitors.

Any cells remaining on the microcarriers at this stage can be removed by gentle agitation. The detached cells can be separated from the microcarriers as described in Section 8.6.1.9. The success of harvesting with trypsin depends on complete removal of medium and serum from the culture and the microcarriers before the trypsin is added (serum contains trypsin inhibitors). pH is critical when harvesting with trypsin, and care must be taken to ensure that harvesting is done between pH 7.4 and 8.0. It is important to expose cells to trypsin for as short a period as possible. For sensitive types of cells, trypsinization at 4°C may be preferable (230), but the advantages of such a procedure must be weighed against the increased time for detachment and the occasional tendency for aggregation. With some types of cell, attachment to Cytodex is very strong, e.g., FS-4 human fibroblasts, and an additional wash with EDTA-PBS is required before the EDTA trypsin is added. Detachment of cells from the microcarriers can be enhanced by continuous stirring in the enzyme solution at a speed slightly greater than that used for normal culture (Section 8.4.3.1).

Trypsin-EDTA solution: The solution can be prepared in Ca^{2+}, Mg^{2+}-free PBS, but the following solution is preferred for retention of maximum cell viability: 122 mM NaCl, 3.3 mM Phenol red, 3.0 mM KCl, EDTA 0.02% (w/v), 1 mM Na_2HPO_4, Tris (hydroxymethyl) aminomethane 2% (w/v), 4.0 mM glucose, pH 7.8–8.0. Trypsin is added to this solution at the usual concentration for a given type of cell. For most cells, 100 mg trypsin/mL is sufficient. Strongly adhering cells, such as FS-4, may require 500 mg trypsin/mL. Trypsin solutions can be sterilized by filtration though a 0.2 mm sterile filter. Since trypsin solutions are subject to self-digestion, it is important to divide freshly prepared solutions into small aliquots and store frozen until required. Crude trypsin solutions have a high content of DNA and RNA (264), and therefore pure recrystallized enzyme is preferred for many biochemical and somatic cell genetics studies. Crude trypsin also shows large batch-batch variation in toxicity, and difficulties with cell growth can often be traced to a specific batch. When possible, test new batches of trypsin for toxicity. The procedures for harvesting cells with Dispase or Pronase are similar to those used for trypsin. The activity of Dispase is not inhibited by serum and thus harvesting must be accompanied by thorough washing of the cells.

Collagenase: Standard cell culture harvesting procedures using trypsin and chelating agents alter cell viability and remove large amounts of surface-associated molecules from the cells (265, 266, 267, 268). If subsequent studies require intact cell membranes, or if rapid harvesting with maximum yields without impairment of cell viability is required, an alternative method of harvesting must be used.

Using collagenase to harvest cells growing on Cytodex 3 is such a method; enzyme digests the culture surface rather than the surface of the cell. Thus, cells harvested with collagenase are generally more viable and have greater membrane integrity than those harvested with trypsin. Harvesting cells from Cytodex 3 with collagenase is the method of choice when using the cells to start cultures at low densities. Collagenase is a proteinase with a high degree of specificity for collagen (269) and can be used for the rapid harvesting of cells from collagen-coated surfaces. For example, Michalopoulos and Pitot (143) reported easy and rapid harvesting of hepatocytes from collagen-coated surfaces, and Sirica *et al*. (144) obtained 100% recovery of rat hepatocytes from a collagen surface within 10 min.

In addition to simplifying the harvesting of cells from cultures, a combination of collagenase and collagen-coated surfaces can be used for the selective removal of different cell types (135, 270). The rate of release of cells from the collagen in the presence of collagenase depends on cell type, with fibroblasts generally being released more rapidly than epithelial cells. The procedure for harvesting cells with collagenase is as follows: Stirring is stopped and the microcarriers allowed to settle. The medium is drained from the culture and the microcarriers washed for 5 min in two changes of Ca^{2+}, Mg^{2+}-free PBS containing 0.02% (w/v) EDTA, pH 7.6 (50 mL PBS/g Cytodex 3). Standard PBS can be used instead for this step if chelating agents are to be avoided. The PBS is removed and replaced by collagenase solution (see below). Approximately 30–50 mL of this solution should be used per gram of Cytodex 3. The microcarriers are then mixed well in the collagenase solution and incubated with occasional agitation at 37°C. After approximately 15 min, the collagenase solution is diluted with fresh culture medium (50 mL medium/g Cytodex 3) and any cells remaining on the microcarriers are dislodged by aspiration with a pipette or by gentle agitation. The detached cells can be separated from the microcarriers as described in Section 8.6.1.9. Collagenase requires Ca^{2+} and Mg^{2+} and therefore chelating agents should not be used during harvesting. When using procedures described in Section 8.6.1.9, steps directed at inactivating collagenase are not usually required. The dilution factor plus cysteine in the medium are sufficient to reduce the enzyme activity. If collagenase must be removed completely, washing cells by centrifugation is the most convenient method.

Collagen solution: Prepare the solution in PBS or Krebs II buffer (163) and sterilize by filtration through a 0.2 mm sterile filter. Collagenase is usually used at a concentration of 100–500 mg/mL.

8.6.1.3 Hypotonic treatment

Incubation in hypotonic solution can be used for harvesting cells that do not have strong adhesion properties, e.g., some established and transformed cell lines. The osmotic shock associated with the hypotonic solution causes the cells to adopt rounded morphology, and they can then be shaken from the microcarriers. Cytodex microcarriers with cells attached are washed twice in hypotonic saline (8 g NaCl, 0.4 g KCl, 1 g glucose in 1 liter distilled water) and incubated in fresh hypotonic saline (50 mL/g Cytodex) at 37°C for 15 min with gentle agitation. Lai *et al*. (125) used hypotonic treatment to harvest CHO cells from Cytodex 1. Cell recoveries are usually less with this method than when enzymes are used. However, an advantage of using hypotonic saline is that harvesting does not involve exogenous protein.

8.6.1.4 Cold treatment

Incubation at low temperatures causes many types of cells to detach from culture surfaces. When Cytodex microcarriers with cells attached are incubated in culture medium without serum at 4°C for 8 h, a significant proportion of cells detach. The sudden fall in temperature associated with a change from warm to cold culture medium often leads to a more rounded cell morphology, and the cells can then be gently shaken from the microcarriers. However, the use of temperature shifts for harvesting cells is generally associated with low viability, and the method is best reserved for established cell lines when other methods are not desirable.

8.6.1.5 Sonication

Sonication alone cannot be used to harvest intact cells from Cytodex microcarriers. However, in combination with the above methods, low intensity sonication can increase cell yields. Sonication can be used to rupture cells and leave membrane fragments attached to intact microcarriers (125; S. Smit, pers. comm., 162).

8.6.1.6 Lignocaine for harvesting macrophages

Some cells are extremely difficult to remove from culture surfaces, e.g., macrophages. Although the methods described above can be used to harvest macrophages, they are usually associated with poor recovery and low viability. An alternative is to use 30 mM lignocaine in PBS (pH 6.7) for 15 min at 22°C (271).

8.6.1.7 Modifications to harvesting procedures for large-scale cultures

The above procedures have been described for small-scale cultures. In principle, exactly the same are used for large-scale cultures although in certain situations modifications may be necessary. Modifications are usually associated with attempts to obtain maximum recovery of cells when processing large volumes of concentrated suspensions of microcarriers. Van Wezel *et al*. (79) describe the use of a trypsinization apparatus for harvesting primary monkey kidney cells from Cytodex 1. This apparatus is based on a Vibromixer (Model El, Chempec. Inc.). Spier *et al*. (272) describe the use of a narrow-bore tube for stripping cells from microcarriers. With a 3.5 cm long capillary tube with a bore of 1.2 mm, greater than 90% of the cells were recovered from the microcarriers (272). Further information on procedures for harvesting cells in specific large-scale culture situations can be obtained from Cytiva.

8.6.1.8 Harvesting from macroporous microcarriers

All reagents and procedures described in the sections above can also be used for Cytopore and Cytoline. Incubation times and protease concentration must be optimized for these microcarriers because the cells grow inside the pores in multilayers. Quantitative removal of cells from a completely dense culture will therefore not be possible; cell loss will be 10% to 30% of the total cells. If the culture is in the exponential growth phase and not confluent, you will get most of the cells out of the pores. The ideal situation for inoculating macroporous microcarriers is to use cells growing in suspension as well. Scale-up strategies are found in Sections 8.6.7 and 8.6.8.

8.6.1.9 Separating detached cells from microcarriers

There are several methods for separating detached cells from microcarriers.

8.6.1.9.1 Differential sedimentation

Recovering cells by differential sedimentation takes advantage of the fact that cells and microcarriers sediment at different rates. For routine harvesting and sub-culturing when maximum recovery is not required, differential sedimentation is the simplest way of obtaining a preparation of cells essentially free from microcarriers. After completing harvesting, culture medium is added (50–100 mL/g Cytodex) and the microcarriers allowed to settle. After approximately 5 min, the culture vessel is tilted to an angle of 45° and the cells collected into the supernatant. Better recovery can be achieved if the microcarriers are washed once more with medium and the supernatant collected. Pooled supernatants can be used directly to inoculate the next culture.

Alternatively, the products of harvesting can be transferred into a narrow container with a high head, for example a test tube or measuring cylinder. After 5 min, the microcarriers settle to the bottom of the container and the cells can be collected in the supernatant.

Using these techniques, it is possible to recover more than 80% of cells in the harvest suspension. A short period of centrifugation (200 g av, 2 min) can be used to hasten sedimentation. If greater recoveries are required, use filtration.

8.6.1.9.2 Filtration

Filtration can be used when it is important to obtain very high recoveries of harvested cells without contamination from microcarriers. Any sterilizable filter with a mesh of approximately 100 μm that is non-toxic for animal cells is suitable (e.g., nylon or stainless steel filters). Sintered glass filters may also be used; however, full recovery of cells may not be possible with such a filter. A convenient filter for small-scale work is the "Cellector" supplied by Bellco Glass Inc. (Vineland, NJ, USA), or similar filter and holder supplied by Cell-Rad (Lebanon, PA, USA). Alternatively, a suitable nylon net can be obtained from Zurich Bolting (Rüschlikon, Switzerland) or Small Parts Inc. (Miami, FL, USA).

8.6.1.9.3 Density gradient centrifugation

Provided a difference in density exists between the cells and the microcarriers, density gradient centrifugation can be used to obtain a preparation of cells free from microcarriers. Manousos *et al*. (98) used discontinuous density gradient centrifugation in Ficoll/paque (density 1.077 g/mL) to achieve efficient separation with no contamination of the cells by microcarriers. Ficoll-Paque™, supplied sterile ready-for-use by Cytiva, can be used for this application.

8.6.1.9.4 Fluidized bed separation

A fluidized bed system can be used to separate cells or virus from microcarriers or microcarrier with cells. The best reactor design would be a conical shape (Fig 83). The fluid stream comes from the bottom and harvest occurs from the top. A top filter with mesh size around 100 µm for Cytodex and Cytopore or 1 mm for Cytoline reduces the risk of losing microcarriers from the system.

8.6.1.9.5 Vibromixer

A vibromixer consists of a disk with conical perforations, perpendicularly attached to a shaft moving up and down with a controlled frequency and amplitude. Increasing the amplitude and the frequency of the translatory movement, increases the turbulence in the reactor. (The shaft is sealed with a membrane or bellows). Vibromixers do not require a dynamic sealing, are a closed system and are therefore considered as very safe regarding containment. Mixing is efficient and gentle (low shear force) Vibromixers have found application in the production of tetanus toxin and animal cell culture.

At larger scale, the bioreactor contents can be emptied into a special harvesting reactor developed by van Wezel (available from B. Braun) (Fig 84). This vessel is divided into two compartments by a stainless steel 60–120 µm mesh filter. The upper compartment contains a Vibromixer, a reciprocating plate with 0.1–0.3 mm holes moving at a frequency of 50 Hz. The microcarriers are collected on top of the mesh. Washing is done by adding washing buffer and draining it through the mesh. Pre-warmed trypsin is added so that it just covers the microcarriers and left for some minutes (depending on the cell line). The Vibromixer is then used for a short while to help detach cells. After detaching, the cells separate from the used carriers by draining through the mesh. Additional washing will improve the yield. The filters work most efficiently with hard carriers (plastic, glass) that do not readily block the mesh. It is also possible to only detach the cells and to transfer the entire mixture of used beads and cells to the new reactor.

8.6.1.10 Measurement of cell viability

Exclusion of dyes provides a convenient measure of cell viability (273). Trypan blue is the dye most commonly used since it can be used with both living material and also material fixed with glutaraldehyde. Trypan blue is the only dye to give reproducible results both before and after fixation. The solution is prepared in PBS (4 mg/mL). Approximately 0.9 mL of diluted cell suspension is mixed with 0.1 mL trypan blue. After 5 min at room temperature, the viable (unstained) and non-viable (stained) cells are counted in a hemocytometer. Counting can be done in conjunction with determination of cell concentration. Staining tests should be performed at pH 7.

Fig 83. A fluidized bed for Cytodex cell separation. Courtesy of Werthenstein Chemie, Switzerland.

Fig 84. Vibromixer harvesting vessel.

8.6.2 Sub-culturing techniques

Sub-culturing cells from one microcarrier culture to another usually involves the steps discussed in Sections 8.4.2, 8.4.3 and 8.6.1. Cells harvested from one microcarrier culture can be used directly to inoculate the next culture containing fresh microcarriers (Fig 30). Transferring a few microcarriers in the inoculum from the previous culture has no effect on subsequent culture development.

8.6.2.1 Colonization

For one sub-culture cycle, it is possible when scaling up to harvest cells from the microcarriers with trypsin, inactivate the trypsin with medium containing serum and then to add fresh micro-carriers. In this way, the culture contains old and new microcarriers. Procedures outlined in Section 8.4.2 are used with corresponding increases in culture volume to maintain a constant concentration of microcarriers. The yield from such cultures is less than that obtained when only new microcarriers are used. It is not possible to use this method when using enzymes to harvest cells from Cytodex 3.

Colonization of macroporous microcarriers (Cytopore 2) by cells propagated on Cytodex 3 for HAV production in a perfusion system was presented by M. Reiter (274) at the IBC conference in Berlin, 2004. Vero cells are grown in roller bottles for inoculation of Cytodex 3. The cells are trypsinized from confluent Cytodex 3 for the inoculation of Cytopore 2 as final production step in 100 liter reactor. With this configuration 2.6 million vaccine equivalents could be produced.

An alternative method for scaling-up is to simply add fresh microcarriers when the culture approaches confluence. Manousos *et al.* (98) demonstrated that adding a further 1 mg of microcarriers/mL of culture prolonged the life of RD cell cultures and improved production of oncornavirus. The success of this method of scaling-up depends on the ability of cells to move from the confluent microcarriers to inoculate the fresh ones. Culture conditions need to be adjusted so that the chance of such a transfer is maximized. In the case of MRC-5 human fibroblasts, static periods of culture with intermittent stirring to avoid aggregation are required before significant inoculation of the new microcarriers can occur (P. Talbot, pers. comm., 128). Reducing the calcium concentration of the culture medium will facilitate transfer of cells between microcarriers (6). Horst *et al.* (127) described the use of Cytodex 1 for sub-cultivation of cells without the use of harvesting procedures. In these experiments, mouse fibroblasts migrated in static cultures from monolayer surfaces onto the microcarriers. More information on proteolytic enzyme-free sub-cultivation can be found in Section 8.6.2.

8.6.2.2 Bead-to-bead transfer

Cultures of cells that do not attach very well to carriers or that detach easily during mitosis can be scaled up just by diluting the microcarrier culture with fresh microcarriers and more media (275). A 1:1000 dilution has been achieved with CHO cells (Fig 86). Microcarriers have been used for protease-free transfer of cells simply by putting them into blood vessels or onto cell culture surfaces and allowing the cells to migrate or crawl onto the surface. The migration method normally works better for transformed cell lines like CHO, hybridomas, etc., (Fig 85) (276).

If transfer just by adding microcarriers does not work, Landauer described a method for enhancing bead-to-bead transfer by releasing a certain portion of cells for recolonization (Fig 87).
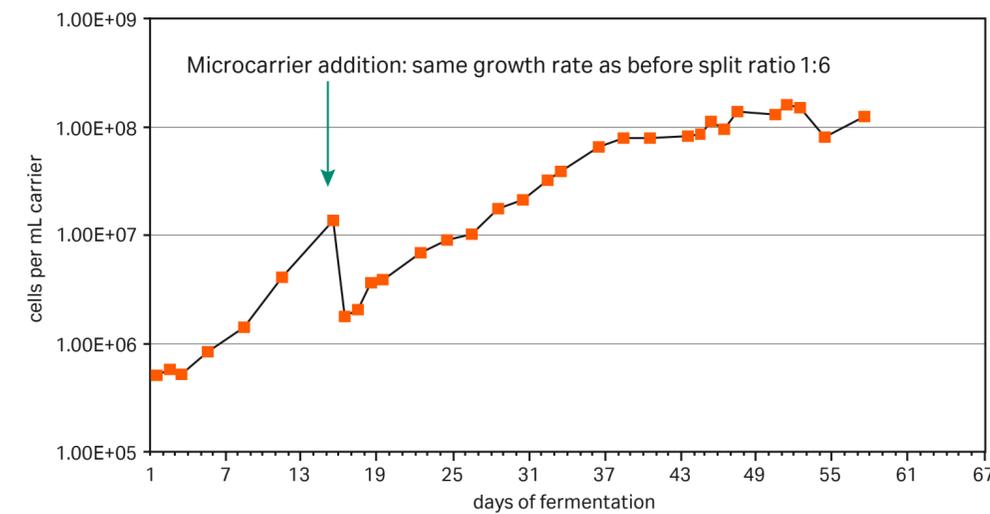
Fig 85. Bead-to-bead transfer in Cytopilot reactor using Cytoline microcarriers. Project financed by Cytiva and performed at the Institute of Applied Microbiology.
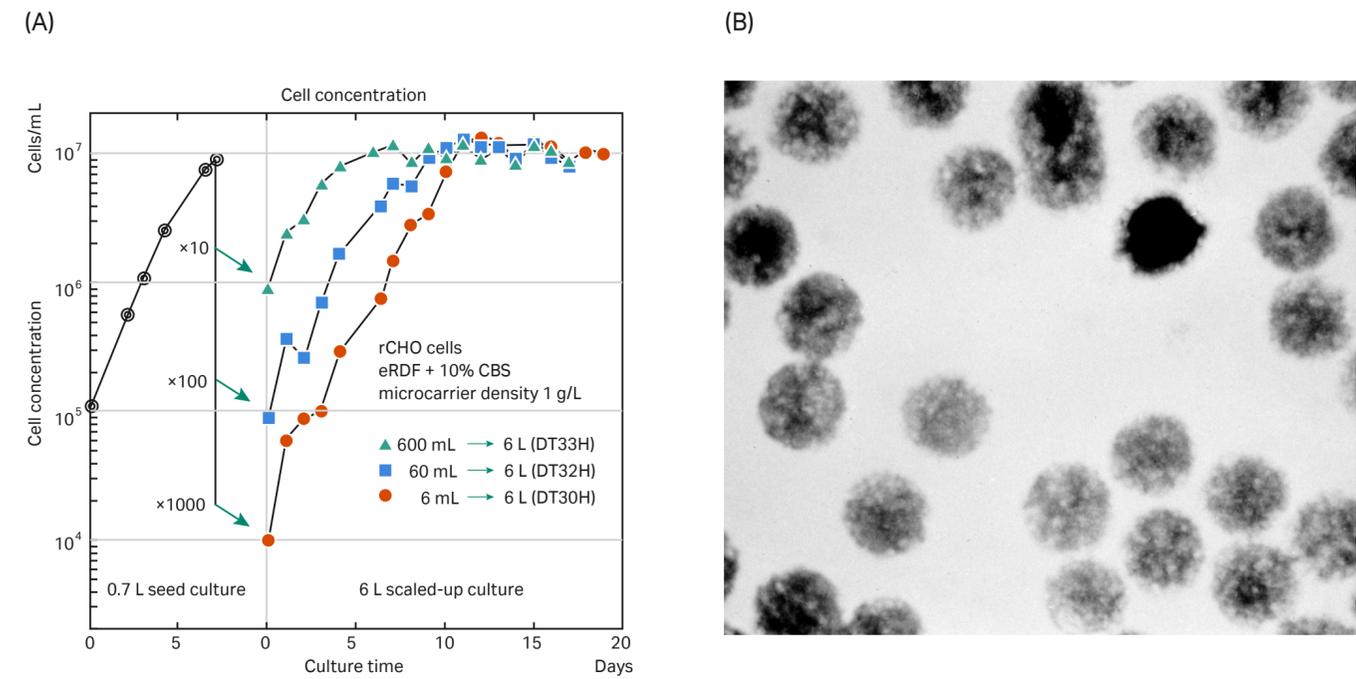
Fig 86. Carrier to carrier transfer (Cytopore) (A) Kinetics. (B) Different phases of colonization, after microcarrier addition.
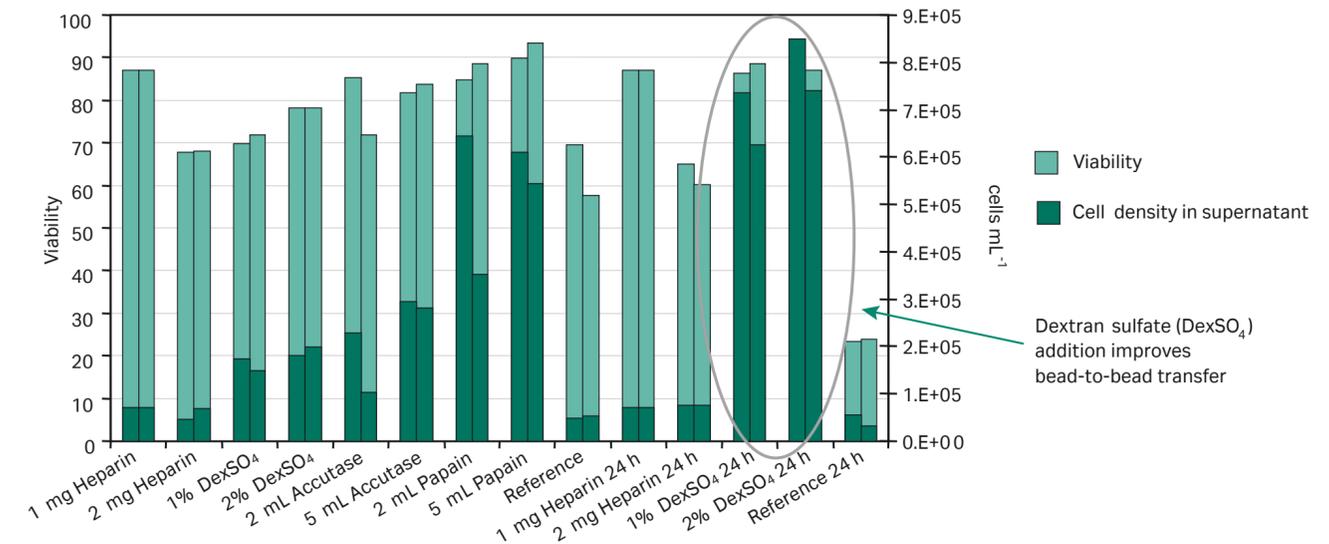
Fig 87. Detachment experiments of CHO cells on Cytoline microcarrier. Viability and cell density are measured. As reference, the standard medium without any further supplementation was used. Source: Karlheinz Landauer *et. al*. Detachment factors for enhanced carrier to carrier transfer of CHO cell lines on macroporous microcarriers (277).

8.6.3 Suspension cells for inoculation

Cells that grow in suspension can first be scaled up by increasing the volume of the suspension cultures. During final production, cells can be immobilized at high densities in carriers and the product continuously harvested via perfusion.

8.6.4 Re-using microcarriers

Using microcarriers for more than one culture/harvest cycle is not recommended. The re-use of surfaces for cell culture requires alternate washing in strongly acidic and basic solutions. These steps are required to remove the debris remaining after the harvesting steps. Such procedures for Cytodex 1 are not recommended since extreme pH may alter both the microcarrier matrix and the degree of substitution. Used microcarriers can be washed in sterile PBS directly after harvesting and used for a further culture step, but attachment of cells is poor and yields are less than 70% of those obtained with fresh microcarriers. Re-use of microcarriers for a third culture step has not been feasible with all cell types tested. For some cell strains, re-using microcarriers is impossible. Cytodex 3 microcarriers cannot be re-used when the cells have been harvested by enzymatic methods. For large-scale production, it is more costly to make a cleaning validation procedure for the re-use of microcarriers than to use new ones.

8.6.5 Waste

Disposal of microcarriers is very much a local issue and costs vary according to country or region. For example, it can be advantageous to dissolve the microcarriers rather than incinerate them.

8.6.5.1 Dissolving microcarriers

Cytodex

It is possible to dissolve Cytodex because it is made of dextran. Two alternatives are:

1. Dextranase
   For smaller scale:
   Dextranase from Sigma (500 U/mg). Use 10 mg Dextranase for 50 mL microcarrier. Incubate at 37°C and pH 6 for 20 min.

   For large-scale:
   Dextranase 50 L from NovoNordisk (Novozymes) with 50 KDU (Dextranase units). Concentration is 5 mL in 1 liter. In our experiments, the concentration of Cytodex was 53 g/L dry weight. Incubation time 2 hours. Temperature and pH (pH optimum is in between 4.5 and 6.0) are optimized but not the other concentrations, stirring and incubation time.

2. Hydrolyze:
   It is possible to hydrolyze Cytodex with strong acids, but not inside stainless steel reactors. Drain the reactor contents first. Neutralize dissolved Cytodex with sodium hydroxide and dispose of the entire amount of liquid.

Cytopore

Theoretically, you can dissolve Cytopore with cellulase, but this microcarrier is so strongly cross-linked that this is hard to do in practice. The concentration of cellulose would be 50 units/mL cellulose solution. Add 50 mL for 20 mL of microcarrier.

8.6.5.2 Incinerating microcarriers

Let the microcarriers sediment, pump them to a collection vessel and send it to a waste treatment plant for incineration

8.6.6 Documentation

The most critical variable in cell culture production is the cell line itself. Therefore, it is essential to have thorough documentation, process control and very strict protocols for all steps from thawing the cells, through small-scale culture, and up to final production. This naturally applies to evaluation studies, scale-up, and process development/ troubleshooting as well. Without this documentation, the reproducibility and quality of the production process will be very poor and the chances of success minimal.

During each phase and step, document:

1. Cells: type, viability, plating efficiency (if attached), doubling time, split ratio at passaging, saturation density (cells/cm^2, cells/mL), yield after harvesting, passage number. Check and keep track of morphology (photographic documentation).

2. Preparation of microcarriers: type, weight/volume, batch, hydration/washing solution and procedures, sterilization, medium equilibration, culture parameter equilibration (pH, dO$_2$, temp.).

3. Culture vessel: type, preparation, media volume, amount of microcarriers used, stirrer speed, air-lift or fluidization rate.

4. Medium: batch, composition, preparation, additives and their batch numbers, buffer system. Serum testing, type, treatment before addition.

5. Gas supply: gases added, batches, set points, flow rates, aids for supply, stirrer, sparging, membrane, filters used.

6. Culture program: inoculation density, preparation of inoculum, inoculation volume, stirring during inoculation, maintenance program, set points, sampling.

7. Results: Cell culture results of each step. Parameter control.

8. Cell harvesting and recovery: washing, chelator treatment, enzyme treatment, viability, yield, enzyme inactivation.

9. Scale-up: separation of carriers, colonization, suspension.

10. Productivity: media change, virus infection, multiplicity of infection, product concentration.

8.6.7 Scale-up methods and process development needs

This section gives an overview of process development needs and examines, in detail, a scale-up method from choosing a cell line to the production process. Process development is concerned with establishing the optimal protocols and equipment that will provide good process economy and efficiency. Establishing the more critical parameters at the beginning of the process facilitates developing large-scale procedures (278, 279).

This scheme only suggests what work is required for process development. It is not a complete suggestion covering all processes (Fig 88).

Development of a microcarrier process


```
Search literature  →  Learn regulations  →  Acquire cell line  →  Culture cell  →  Check for contaminants  →  Characterize the cell  →  Select cell, transfect
                       (FDA, WHO)            (ATCC, ECACC, Lab, Isolate)                                                                    (optimize productivity)
```

```
Optimize culture conditions  →  Generate Master Cell Bank  →  Generate Working Cell Bank  →  Select microcarrier  →  Process simulation  →  Fermenter (bench)  →  Pilot scale  →  Production
Check growth characteristics                                                                 (in stationary culture)   stationary, spinners   Reactor optimization   Process optimization
        1                              1                                                              2                       3                      4                    5              6
```

Fig 88. Schematic of the development of a microcarrier process.

Process simulation is done to ascertain that the cells will manage the number of steps needed to reach final production volume with required productivity
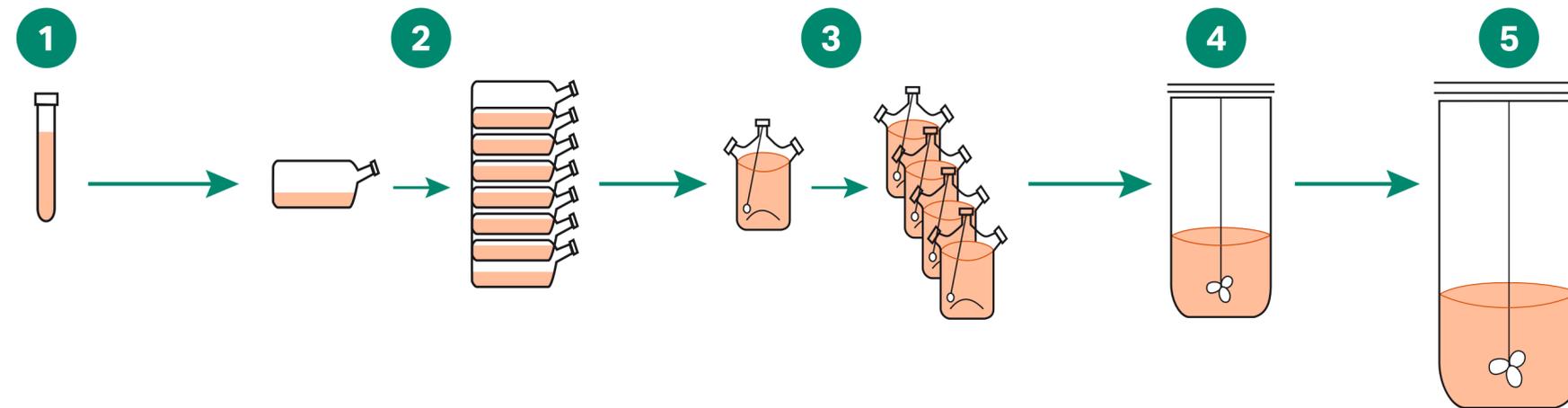
Process simulation
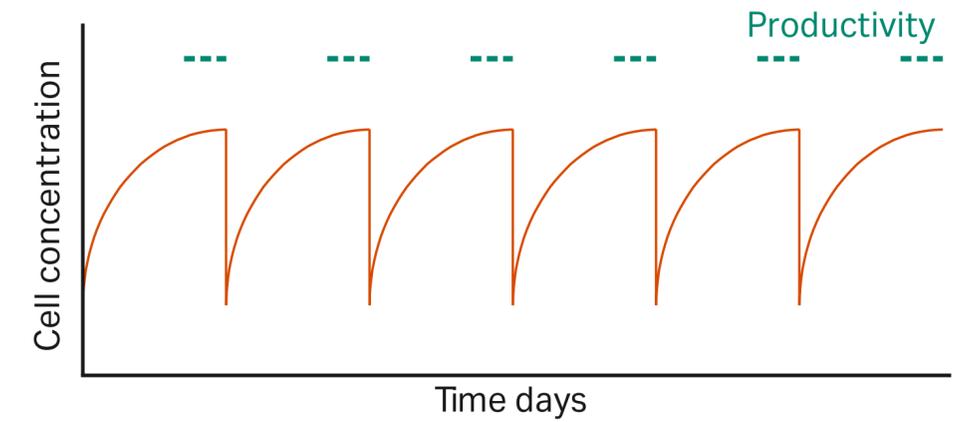
1

Suggested experiments in flasks (rollers):

- Viability, plating efficiency of thawed cells
- Optimal inoculation density (maximal cell doublings)
- Growth kinetics
- Harvesting protocol (single cell suspension, optimal density for harvesting, cells in cell cycle)
- Yield, viability, plating efficiency of the harvested cells
- Productivity (final titer, per cell basis, reference under stationary conditions)

2

Suggested experiments in stationary culture (bacterial Petri dishes)

- Choice of carrier
- Preliminary tests of suitable bead concentration (batch)
- Optimal seeding density (cell/bead ratio, plating efficiency)
- Optimal pH during attachment phase
- Serum screening
- Cell attachment, cell spreading tests
- Media formulation tests
- Suitable dO_2 concentration
- Subcultivation/migration tests

3

Suggested experiments in spinner flasks

- Attachment efficiency in stirred culture
- Growth curves, rate
- Appropriate microcarrier concentration, optimal inoculation concentration
- Starting conditions, initial volume, static attachment period, stirring speed
- Check media pH (too alkaline, bad attachment)
- Determine media replacement strategy
- Check productivity under stirred conditions
- Optimize harvesting/migration procedure (stirrer speed, washing, buffer capacity, trypsin (enzyme conc. U/cell) incubation time, pH)
- Check viability, recovery, attachment efficiency when harvesting different times after inoculation
- Process simulation

4

Suggested experiments in bench fermenter

- Attachment efficiency
- Optimize culture parameters, pH, dO_2, stirring speed
- Appropriate microcarrier concentration, optimal inoculation concentration, high density and perfusion
- Starting conditions, initial volume, static attachment period, stirring speed (other geometry)
- Determine media replacement strategy, perfusion
- Develop microcarrier retention technology, perfusion culture
- Develop harvesting technology from reactors

5

Suggested experiments in pilot scale

- Alternative procedure for carrier preparation, due to volume, inside fermenter (hydration, washing, sterilization, surface activation (glass 0.5 M NaOH))
- Evaluate sedimentation rate, culture control during settling for media change, reduction of volume
- Develop technology for harvesting (manually not feasible)
- Develop inactivation procedures
- Develop cleaning procedures

6

Last but not least:

- Qualification
- Validation
- Registration

Scale-up issues on:

Surface carriers, in stirred tanks

- Already discussed
- A well established and proven technology running in 1000 L scales in a number of places

Macroporous carriers, in stirred tanks

- The main issue is harvesting of a single cell suspension, of anchorage-dependent cells
- Otherwise the same concerns as for surface carriers
- Carriers suitable for semi adherent and suspension cells

Macroporous carriers, in fluidized beds

- The main issue is harvesting of a single cell suspension, of anchorage-dependent cells
- Carriers are bigger, handling
- Scale up potential of technology
- Otherwise the same concerns as for surface carriers
- Carriers suitable for semi adherent and suspension cells

Macroporous carriers, in packed beds

- The main issue is heterogeneous culture
- Harvesting of a single cell suspension, of anchorage-dependent cells
- Carriers are bigger, heavier, handling
- Otherwise the same concerns as for surface carriers

3D aggregates

- Difficulty of controlling aggregate size, sedimentation
- Heterogeneous culture
- Harvesting of single cell suspension
- Otherwise the same concerns as for surface carriers

Vaccine considerations

- Access to cell upon infection
- Possibility of media change, carrier concentration upon infection
- Culture control
- Retrieval of virus particle
- Retrieval of intact cells
- Secreted rec-vaccines (other technology choices)

8.6.8 Integrated bioprocessing

This section describes an example of efficient monoclonal antibody production by integrated bioprocessing. It shows how investing in your upstream process makes your downstream process easier and cheaper. As described in Section 1.1, the cell load in the supernatant is much lower than with batch and fed-batch cultures. You are therefore able to use expanded bed technology, big beads or filtration to make the clarification procedure much faster and easier (Fig 89).

Stirred tank fed-batch

Cytopilot fluidized bed continuous perfusion

Buffer vessel

Expanded bed affinity purification
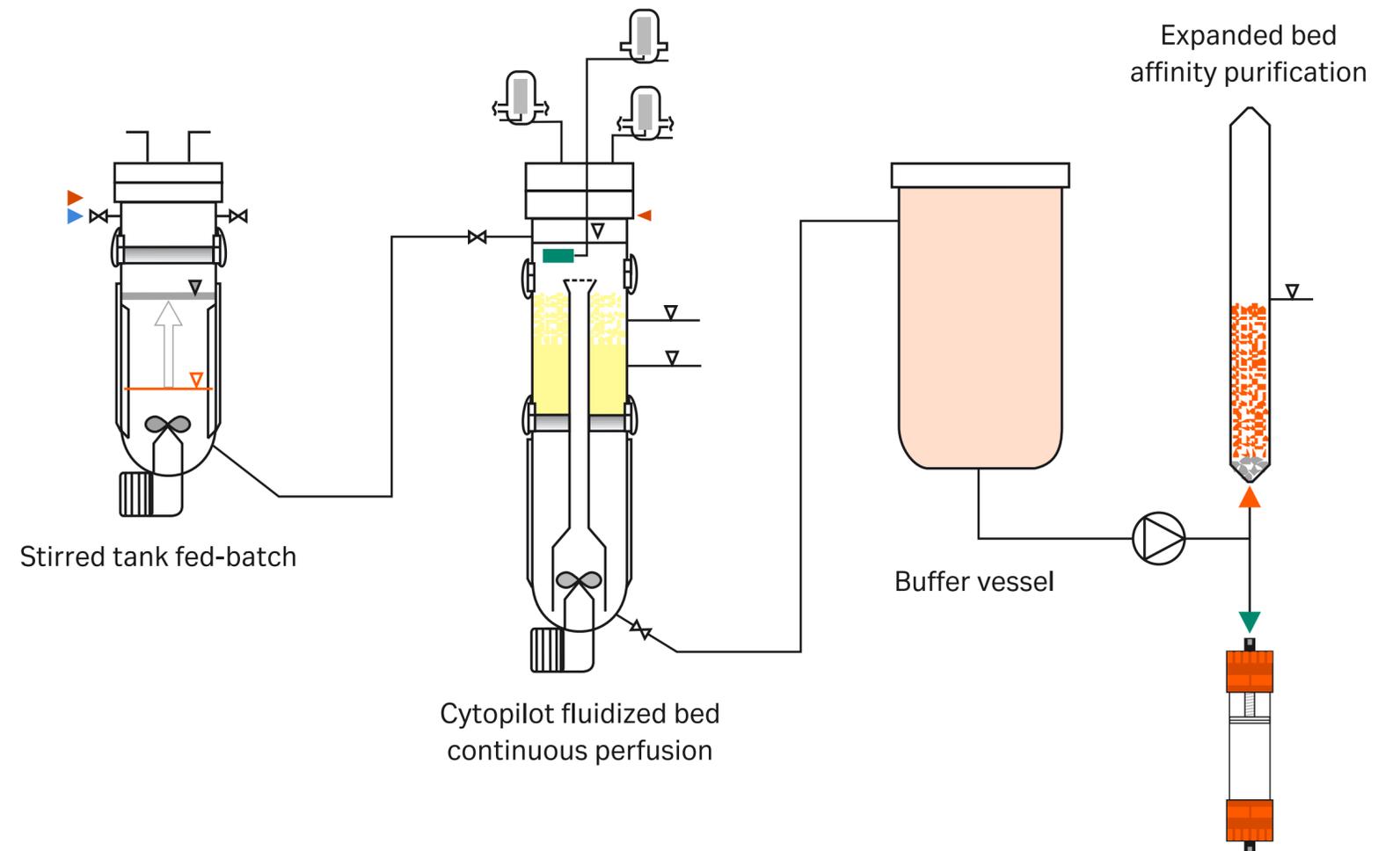
**Fig 89.** Outline of a production scheme for Mab production using stirred tank for inoculum preparation and Cytopilot fluidized bed as production unit. Low cell numbers in the supernatant guarantees the optimal performance of the expanded bed (Streamline) with protein A affinity for clarification and purification in one step. Courtesy IAM; Polymun Scientific GmbH.

09

Production considerations

9.1 Production economy

Calculating total production economy is complex. Many aspects need to be considered: the organism to be cultured, amount of product needed, medium volumes, equipment, staff, downstream costs, etc. The major part of the Cost Of Goods Sold is, however, fixed costs (280). Investments in equipment and personnel thus determine whether or not a project will be profitable.

Ideally, investments should be suitable for other projects if the one planned is not successful. Sometimes the choice of technology is restricted. For example, batch/fed-batch processes must be used when producing cytopathic viruses. To maximize the benefits of perfusion, immobilization is also needed to prevent the washout of cells. The cost ratios for perfusion, continuous-flow and batch techniques to produce monoclonal antibodies are 1:2:3.5 (281, 282, 283).

9.1.1 Consumable cost comparison

The three major consumables are the culture surface (for anchorage-dependent cells), serum (or protein additives), and medium. The medium cost can normally not be affected other than by negotiating price with different suppliers.

Culture surface

To make a true cost comparison of cell culture surfaces, grow a specific cell line on different supports and calculate the cost of each support/yield of 10^6 cells. Start by calculating the price/m^2 of material. Take care when comparing porous materials. Some determinations may also measure surfaces inside pores not accessible for cells. The true cell culture surface will thus be smaller than that measured. Remember that the larger the diameter of cell, the smaller the accessible area. Large cells thus give much lower densities in macroporous carriers.

As large a surface area as possible is desirable as it allows a higher number of cell doublings. With a large surface area and an optimized inoculation density, it is possible to maximize the cellular multiplication in each culture step. This reduces the number of scale-up steps and equipment necessary to reach final production volume (cell number).

Comparing cost of surface area/yield of 10^6 cells reveals the following. Microcarriers and the Cell Cube (Costar) have the same order of magnitude and the lowest cost, roller bottles (1400 cm^2) are five times more expensive, and hollow fiber reactors 200 times more expensive in generating the same number of cells. (The cell density used for the hollow fiber is that given by the supplier.)

Serum and additives

The most expensive additive is serum (proteins). Reducing serum in the medium by 1% lowers costs by approx. 3 USD/L.

Comparing the costs of a suspension culture with a fluidized bed run reveals the following. (It is assumed that serum concentration can be reduced by 1% due to the higher cell density in the fluidized bed — this is a modest assumption.) Bed volume is 5 L. This is normally perfused with 10 volumes of medium/bed volume/day = 50 L/day. The saving is then 3 × 50 = 150 USD/day. If run for 30 days, the total saving is 4500 USD. Subtracting the cost of the carriers gives a net saving of 3740 USD/run. A 5 liter fluidized bed reactor with perfusion thus produces the same amount of cells and products in 1 month as a 1500 liter batch reactor does in 1 week at a consumable cost saving of 3740 USD. The investment cost is also much lower for the smaller system.

Table 21 compares the medium utilization (mL spent medium/mg product) of a human hybridoma cell line in a continuous stirred tank reactor (CSTR) with a fluidized bed reactor.

Table 21. Medium utilization in continuous stirred tank and fluidized bed

	CSTR	FBR
Culture volume	20 L	20 L (10 L of carrier)
Dilution-Perfusion rate (L/h)	0.0125	0.105
Medium L/day	6	50
Product (mg)/day	50	400
mL spent medium/mg product	**120**	**125**

9.1.2 Volumetric productivity comparison

Many parameters can be used to compare different production systems: product concentration, gram/day, specific productivity, total grams per run, etc. Not all are useful because, for example, the size of the reactor, batch or continuous alternatives, length of run, etc., are not always considered (284). However, volumetric productivity does consider size of the reactor, time and productivity. Volumetric productivity is the amount of product per liter of reactor volume and day. Using this parameter, it is easy to see the efficiency of the system. Mahadewan (284) claims that if you set the relative antibody production rate for the continuous perfusion model to 100%, batch will reach only 9% and fed-batch 42% of the value of the perfusion technology.

9.1.2.1 Batch, fed-batch versus perfusion with macroporous microcarrier

Figure 90 shows different system operations. Perfusion is 10 times more efficient than batch technology or the perfusion reactor can be 10 times smaller than the batch reactor (285, 286, 287, 288, 289, 290). Nutrients are depleted throughout the run and waste products (DNA, host cell protein, etc.) are accumulated in batch operations (Fig 91), which has a big influence on product quality throughout the run (Section 9.2). With perfusion technology, nutrients and by-products remain constant over the entire period. Perfusion technology mimics the bloodstream inside the body.

The reduced downtime (Fig 92) of perfusion technology also makes this mode of operation much more cost effective; fewer personnel are needed.

The efficiency of perfusion with macroporous microcarriers is demonstrated in the following calculation using the stirred tank fed-batch data from Section 4.3, where the output of a 15 000 liter stirred tank is an average of 170 kg of antibody per year.

If an average production with a perfused fluidized bed (little bit lower with a stirred tank with Cytopore) of 1 g/liter carriers and day is the base for the calculation, you are able to produce 2 kg/day with a fluidized bed of 2000 liters of Cytoline. The working volume of the reactor would be 5000 liters. A normal run time of 100 days gives 200 kg of antibody. You are able to run this fermenter three times a year, which means 600 kg of antibody for therapy. This calculation showed a 3-fold greater production with 1/3 of the volume.
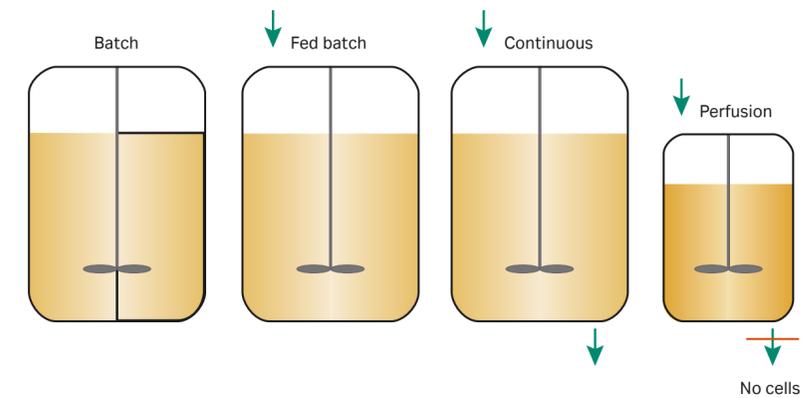

**Fig 90.** Stirred tank reactor in different operation modes. Batch, fed-batch, continuous, perfusion.
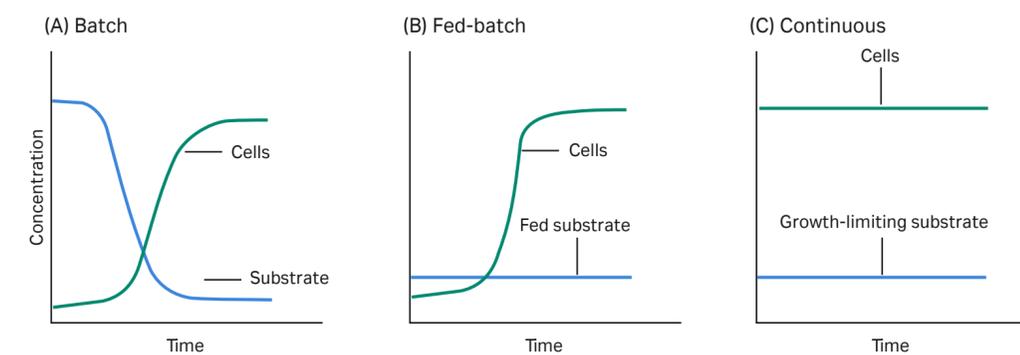
**Fig 91.** Comparison of batch and perfusion concerning nutrients and waste products.
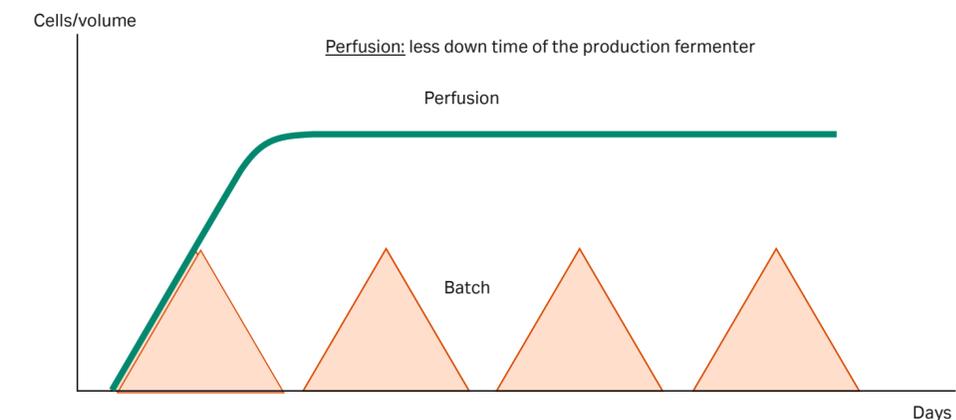
Fig 92. Comparison of batch and perfusion concerning down-time of the fermentation plant.

9.1.2.2 Roller bottle versus macroporous carriers (fluidized bed technology)

The following calculations are based on a human hybridoma cell line producing human anti-HIV1 monoclonal antibody, specific productivity around 10 µg/10^6 cells and day. This is a low producer compared with murine hybridomas, but about the average for a human xeno hybridoma.

Roller bottle: 500 mL suspension in 850 cm^2 bottle
                      repeated batch
                      1.25 mg IgG/day

Fluidized bed: 500 mL macroporous carriers (Cytoline 1)
                      perfusion culture
                      50 mg IgG/day

= 40-fold productivity per day from the fluidized bed over roller bottles

These results are based on actual experiments!

The carrier load in the fluidized bed fermenter is normally 40% of the reactor volume. This means that the output based on the reactor volume is 20 mg IgG/reactor and day. That is still a 16-fold productivity increase compared with roller bottle technology.

9.1.2.3 Roller bottles versus Cytodex

The advantage over roller bottle production of, for example, vaccine production is clear: less handling and a homogeneous culture. The capacity of 4000 roller bottles is obtained from one 500 liter stirred tank reactor using 5 g of Cytodex (Fig 40).

9.1.3 SPF egg production versus Cytodex

At the Williamsburg conference (1998), Ruud Brands presented a successful development of a tissue-culture-based, MDCK-derived influenza vaccine (89). The advantages of the microcarrier alternative to the classical egg-derived vaccine were clearly pointed out. With the MDCK cell culture-based influenza vaccine, it is possible to increase vaccine production over time. In addition, production occurred in serum-free medium. Clinical data showed that the vaccine was safe and well tolerated.

9.1.4 Low cost production of rabies vaccine

In many parts of the world (Latin America and Asia), rabies kills 30 000 people per year. Rabies is preventable and curable but many countries are often not able to treat the people with expensive medicine. In an article in Genetic Engineering News in 2002, Eduardo Aycardi describes a possibility to produce 1 million doses of rabies vaccine per year using a 30 liter bioreactor. The process uses 25 g/L of Cytodex and requires just three lab technicians working in a 350 m^2 facility under GMP requirements.

9.1.5 Size of the production plant

Roller bottle: If you have a good and productive murine hybridoma cell line (100 µg/10^6 cells and day) you will get:

12.5 mg IgG/day

800 roller bottles of 500 mL running for 100 days produce 1 kg. You have to manipulate 800 rollers a day. Also, you need a large incubator room for the rollers if you want to produce 1 kg of product.

Fluidized bed: The product output per liter carriers and day would be 1 g. If you run 100 days, like the roller culture above, you would need a 10 liter carrier bed. The reactor size would be around 25 liters. The height of this reactor is 0.91 m and the diameter 0.2 m. The complete reactor with all components in a rack (valves, filters, heating circuit, etc.) is 1.8 m high, 0.9 m broad and 0.54 m deep (see brochure from Vogelbusch).

If you want to shorten the running time, use a 100 liter reactor with a 40 liter bed. The time of fermentation would be around 25–30 days. The size of this reactor is 1.5 m high and 0.3 m diameter. The complete reactor with all components in a rack would be 2.5 m high, 1.1 m broad and 0.7 m deep.

These figures are based on the above experiments but calculated with a higher specific productivity (assumption).

9.2 Product quality

The dynamics of cell growth (291), cell density, productivity, titer and production of recombinant proteins or monoclonal antibodies are not always the most important factors for producing a pharmaceutical. Product quality is a very important issue. If the fermentation system does not result in an intact, fully glycosylated product, a high productivity system can turn out to be very expensive because of the very high downstream costs needed to purify the desired protein. The more you invest in optimizing your culture upstream, the more you save on downstream processing.

As shown in Section 9.1.2.1, it is important to provide a constant environment for the production of cells by using perfusion technology (292).

Goldman *et al.* (293) showed that the sialylation of glycans of recombinant human IFN-gamma was consistent throughout the fluidized bed perfusion culture, whereas sialylation declined significantly in the stationary and death phase of a comparable batch culture. Wang *et al.*, 2002 (294), showed that the glycosylation profile of EPO from perfusion culture with macroporous beads did not differ from other culture systems. EPO may be vulnerable to any limitation during culture because of its extensive level of glycosylation. From observed electrophoretic patterns, there was clearly no significant difference in the profiles from the products of perfusion culture compared with those from stationary and suspension culture.

9.3 Important developments

Continuous development of manufacturing technology is needed if animal cell culture processes are to be competitive. Better cell culture, higher productivity and simpler media requirements are all important (280). Yields should increase in relation to space used. Processes should be robust with high success rates and minimum down-times. The number of operations should be as few as possible. Plants should be multi-purpose to fully utilize capacity. Remember that fixed costs make up most of the Cost Of Goods Sold, and that R&D normally focuses on work that affects variable costs!

10
Optimizing culture conditions

The quickest way to determine the key variables in a culture is to set up factorial design experiments. Feed a limited number of experimental results into mathematical models and evaluate the variables that require attention and further optimization. The fewer the cell lines, the more in-depth the optimization that can be done.

However, each recombinant construct has different growth properties and requirements. To fully optimize media utilization, analyze the amino acids and find out what factors in the medium are limiting. These factors can be added selectively or the medium composition changed to better suit the cell type. If there is no time or resources for this analysis, use a surplus of medium to maintain steady state conditions and maximize growth rate.

11
Troubleshooting

11.1 Stirred microcarrier cultures

Some problems may arise when working with stirred microcarrier cultures for the first time. The following list summarizes typical areas of difficulty and the most likely solutions. These points also form a useful checklist when culturing new types of cells.

1. **Medium turns acidic when microcarriers added**

   - Check that the microcarriers have been properly prepared and hydrated

2. **Medium turns alkaline when microcarriers added**

   - Gas the culture vessel and equilibrate with 95% air, 5% CO_2

3. **Microcarriers lost on surface of culture vessel**

   - Check that the vessel has been properly siliconized

4. **Poor attachment of cells and slow initial growth**

   - Ensure that the culture vessel is non-toxic and well washed after siliconization

   - Dilute culture in PBS remaining after sterilization and rinse microcarriers in growth medium

   - Modify initial culture conditions, increase length of static attachment period, reduce initial culture volume or increase the size of the inoculum

   - Check the condition of the inoculum and ensure it has been harvested at the optimum time with an optimized procedure

   - Eliminate vibration transmitted from the stirring unit

   - Change to a more enriched medium for the initial culture phase

   - Check the quality of the serum supplement

   - If serum-free medium is used, increasing attachment protein concentration may be necessary (fibronectin, vitronectin, laminin)

   - Check for contamination by mycoplasma

5. **Microcarriers with no cells attached**

   - Modify initial culture conditions, increase length of static attachment period, reduce initial culture volume

   - Improve circulation of the microcarriers to keep beads in suspension during stirring

   - Check the condition of the inoculum, especially if it is a single cell suspension

   - Check that the inoculation density is correct (number of cells/bead)

6. **Aggregation of cells and microcarriers**

   - Modify initial culture conditions, reduce the time that the culture remains static

   - Increase stirring speed during growth phase, improve circulation of microcarriers

   - Reduce the concentration of serum supplement as the culture approaches confluence

   - Reduce the concentration of Ca^{2+} and Mg^{2+} in the medium

   - Prevent collagen production by adding proline analogs to the culture medium

7. **Rounded morphology of cells and poor flattening during growth phase**

   - Replenish the medium

   - Check the pH and osmolality of the culture medium

   - Reduce the concentration of antibiotics if low concentrations of serum are used

   - Check for contamination by mycoplasma

8. **Rounding of cells when culture medium is changed**

   - Check temperature, pH and osmolality of replenishment medium

   - Reduce the serum concentration

9. **Cessation of growth during culture cycle**

   - Replenish the medium or change to a different medium

   - Check that pH is optimal for growth

   - Re-gas the culture vessel or improve supply of gas.

   - Reduce stirring speed

   - Check for contamination by mycoplasma

10. **Difficulties in controlling pH**

    - Check that the buffer system is appropriate

    - Improve the supply of gas to the culture vessel, lower the concentration of CO_2 in the headspace or increase the supply of oxygen

    - Improve the supply of glutamine, supplement the medium with biotin or use an alternative carbon source, e.g., galactose

11. Difficulties in maintaining confluent monolayers

- Check that pH and osmolality are optimal

- Reduce the concentration of serum supplement

- Improve the schedule for medium replenishment

- Reduce the concentration of antibiotics

- When culturing cell lines that produce proteases in a serum-free medium, it may be necessary to add protease inhibitors to prevent the cells from detaching (CHO cells have been shown to secrete proteases!)

12. Broken microcarriers

- Ensure that dry microcarriers are handled carefully

- Check the design of the culture vessel/impeller and ensure that the bearing is not immersed in the culture

13. Difficulty in harvesting cells from microcarriers

- Ensure that the carriers have been washed extensively together with mixing

- Check that approximately the same amount of protease (U/cell) is used as when harvesting from flasks

- Check that the trypsin has not been thawed for too long (loss of activity)

- Check that sufficient shear force is used in addition to trypsinization

14. Microcarriers float in foam due to sparging

- Reduce the serum/protein concentration as much as possible

- Add pluronic F68 to decrease foaming

- Add polymers to increase viscosity

- Aerate via silicone tubing (Diesel, bubble-free aeration), via spin filter (New Brunswick, Celligen), via external loop (vessel, hollow fiber)

11.2 Fluidized bed

1. **Culture medium too acidic**

   - Expel CO_2 by using a sparger that creates large bubbles

   - Add sodium hydroxide to titrate the pH, observe the osmolarity

   - Try to increase buffer capacity

   - Optimize the culture medium and oxygen support to avoid production of lactic acid

2. **Bridging of microcarriers**

   - Use higher circulation rates. This shortens contact time between the microcarriers. Bed expansion should be between 150% and 200%.

3. **No attachment**

   - Initial cell density too low

   - pH level and dO_2 concentration were incorrect during inoculation

   - Contact time between cells and the microcarriers was too short. More time is needed in packed bed mode.

   - Microcarriers were not washed properly

   - Microcarriers were not equilibrated

   - Cell inoculum was in stationary phase and not in the exponential growth phase

4. **Oxygen supply too low**

   - Use microsparging technique with a sparger that creates small bubbles (pore size around 0.5 μm)

   - Use pure oxygen for gassing. Cells inside the pores of the macroporous microcarriers are protected against the toxicity of oxygen.

   - Increase the circulation rate

12
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