Microcarrier cell culture

principles & methods

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Background

1.1 Introduction

Cell culture techniques have become vital to the study of animal cell structure, function and differentiation an for the production of many important biological materials such as vaccines, enzymes, hormones, antibodies, interferon's 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. 1) which are usually suspended in culture medium by gentle stirring. By using microcarriers in simple suspension culture systems it is possible to achieve yields of several million cells per milliliter.

Cytodex® microcarriers have been specifically developed by Pharmacia Biotech for the high yield culture of a wide range of animal cells (section 6.1) in culture volumes ranging from a few milliliters to several hundred liters. The special requirements of the microcarrier system (section 2.1) are best fulfilled by the dextran-based beads which are subsequently derivitized to form the three types of Cytodex microcarriers.

- The surface characteristics of the microcarriers have been optimized for efficient attachment and spreading of cells.
- The size and density are optimized to facilitate even suspension and give good growth and high yields for a wide variety of cells.
- The matrix is biologically inert and provides a strong but non-rigid substrate for stirred microcarrier cultures.
- The microcarriers are transparent and allow easy microscopic examination of the attached cells.

Experience with Cytodex for a wide variety of applications has confirmed the importance and benefits of the microcarrier technique.

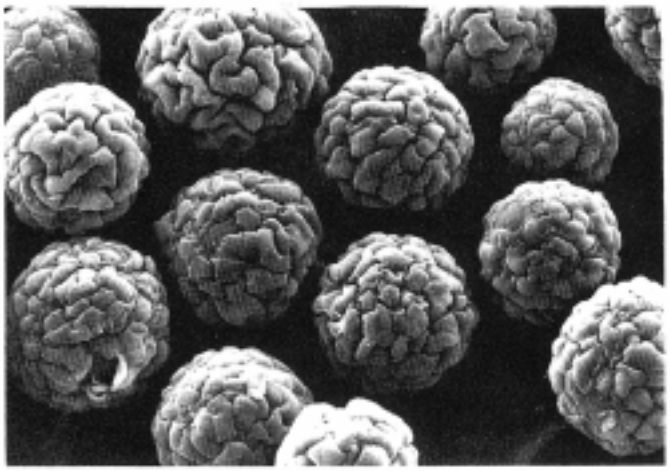


Fig. 1. Scanning electron micrograph of pig kidney cells (IBR-S2) growing on Cytodex 72 h after inculation. (Original photograph by G. Charlier, INVR, Brussels, Belgium, reproduced by kind permission.)

New opportunities and applications for animal cell culture
Cytodex provides convenient surfaces for the growth of animal cells and can be used in suspension culture systems or to increase 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 labelled compounds (see section 1.4. for a description of these applications).

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 than other types of monolayer cultures. The possibility to culture cells in small compact culture systems is especially important when working with pathogenic organisms.

Improved control
Suspension culture systems provide excellent opportunities for the control of culture parameters (e.g. pH, gas tensions etc). The microcarrier technique provides a method for growing anchorage-dependent cells in a system having all the advantages of suspension culture. The improved control possibilities with microcarrier culture allow for a homogenous culture system having 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.

Reduced requirements for culture medium
When compared with other monolayer culture techniques stirred microcarrier cultures yield 2-4 times as many cells for a given volume of medium. The superior yields with microcarrier culture 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 reduction in requirement for medium means considerable savings in cell culture costs (6,9), particulary when expensive serum supplements such as foetal calf serum are used.

Reduced requirements for labour
Because large numbers of cells can be cultured in small volumes (more than 10^9 cells/litre) fewer culture vessels are required when working with microcarrier cultures. For example, with microcarrier culture one technician can handle a vaccine production equivalent to 900 roller bottles per week (10). One litre of microcarrier culture can yield as many cells as up to 50 roller bottles (490 cm^2 bottles, 2).
The simplified procedures required with microcarriers reduce the labour necessary for routine production and save on cleaning and preparation of glassware.
Separation of cells from the culture medium is simple; when the stirring is stopped the 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.

Lower risk of contamination
In cell culture the risk of contamination is related to the number of handling steps (opening and closing of culture vessels) required to produce a given quantity of cells or their products. Microcarrier culture provides a method for reducing the number of handling steps. There is a much reduced risk of contamination when the production of large quantity of cells is from a single microcarrier culture rather than several hundred roller bottles (6).

The principles and methods necessary to achieve the best results with microcarrier culture are described in this book. Although this technique is one of the most advanced in animal cell culture it need not be restricted to experienced cell culturist. Since cell culture is being used by a wide variety of scientists this book is written for both beginners and those experienced in cell culture and only a basic knowledge of cell culture is assumed.

This book aims at describing the principles and techniques of cell culture with Cytodex so that the reader is able to deduce optimum procedures with a minimum of effort. The principles aim at a flexible and systematic approach. They are essential to making the most off microcarrier culture and to achieving consistent results with high yields.

All methods described her have been developed for use with Cytodex and are not necessary suitable for use with other surfaces for cell culture.

1.2 Adhesion of cells to culture surfaces

The adhesion of cells to culture surfaces is fundamental to both traditional monolayer culture techniques and microcarrier culture. Since the proliferation of anchorage-dependent cells can only occur after adhesion to a suitable culture surface (11), it is important to use surfaces and culture procedures which enhance all of the steps involved in adhesion. Adhesion of cells in culture is a multistep 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 (11, fig. 2).

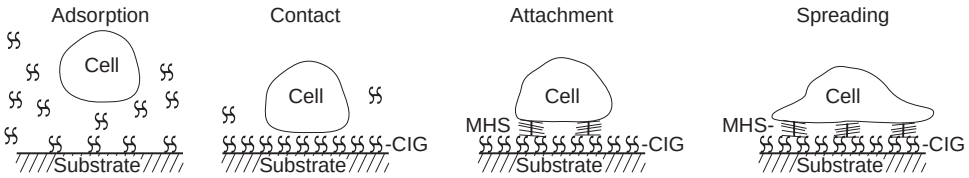


Fig. 2. Simplified outline of steps involved in adhesion of animal cells to culture sufaces. The whole process involves divalent cations and glycoproteins adsorbed to the culture surface. Under usual culture conditions the attachment proteins vitronectin and fibronectin originates from the serum supplement in the medium. MHS is synthesized by the cells. CIG - fibronectin or vitronectin. MHS – multivalent heparan sulphate. (Adapted from refs. 11, 17, 30).

The culture surface must be hydrophilic and correctly charged before adhesion of cells can occur (11). All vertebrate cells possess unevenly distributed negative surface charged (12) and can be cultured on surfaces which are either negatively or positively charged (11,13-16). 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 tather than the polarity of the charges (15,17).

Two factors in culture medium are essential for adhesion of cells to culture surfaces - divalent cations and protein(s) in the medium or adsorbed to the culture surface (11). In the absence of protein and divalent cations cells attach to a culture surface only by non-specific adsorption (11,18). The protein molecule essential for full adhesion of cells to a culture surface is now known to be a glycoprotein (19-21). The "critical charge densities" noted for microcarriers (16,22-24, fig 4) and other culture surfaces (14) are more likely to be related to interactions between attachment glycoprotein(s) and the charged surface rather than direct electrostatic interaction between the cells and the culture surface (17).

Attachment glycoproteins found in the serum in culture medium are fibronectin and vitronectin, secreted from certain cells (19,25-27). Vitronectin and/or fibronectin must be adsorbed on the culture surface before they can promote cell attachment and spreading (28) and they are subsequently incorporated into the extracellular matrix of the spread cells (27). Under normal culture conditions multivalent heparan sulphate proteoglycans mediate adhesion of cells to culture surfaces by co-ordinate binding to glycoproteins on the cell surfaces and the CIG adsorbed on the culture surface (20).

In order to achieve good adhesion of the cells to culture surfaces it is necessary that the requirement for an attachment glycoprotein is satisfied. Many established and transformed cell types secrete only very small amounts of fibronectin and require a fibronectin or serum supplement in the culture medium before adhesion occurs (18,25). Certain types of cells such as diploid fibroblasts can secrete significant quantities of fibronectin and do not require an exogenous source of this glycoprotein for attachment (19,29).

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) foetal calf serum contains approximately 2-3 µg fibronectin/ml (27) and a large proportion of the fibronectin adsorbs to culture surfaces within a few minutes (18). Serum-free media often require addition of fibronectin (1-50 µg/ml) before many cells can attach to culture surfaces. A minimum of 15 ng of adsorbed fibronectin/cm^2 is required for spreading of an established type of cell, BHK (25). Therefore, standard culture procedures usually ensure that the culture surface (plastic, glass or Cytodex microcarrier) is coated with adequate amounts of glycoproteins involved in cell attachment.

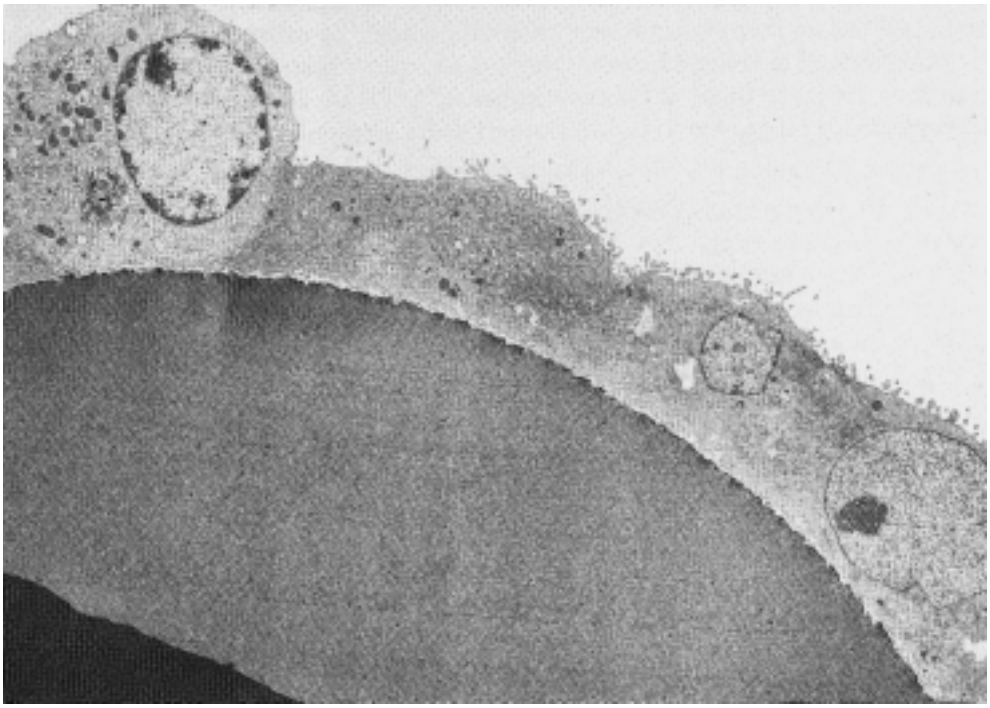


Fig. 3. Transmission electron micrograph of pig kidney cells growing on a Cytodex microcarrier. (Original photograph by B. Meigneir and J. Tektoff, IFFA-Mérieux. Lyon, France, reproduced by kind permission.)

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 the steps of adhesion (fig. 2) are fully completed. The way in which microcarrier culture procedures are designed 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 3 and 5. Figure 3 illustrates the close attachment of cells to Cytodex.

1.3 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* (31). In the first experiments *van Wezel* (31) used the beaded ion-exchange gel, DEAE-Sephadex® A-50 as a microcarrier. This type of microcarrier 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 for suspension which were not compatible with cell growth (17,31). Several workers have suggested that the ideal microcarrier should have properties similar to those of DEAE-Sephadex A-50 (16,22,32-35). Other ion-exchange beads all proved to be inferior to DEAE-Sephadex A-50 (13,14,16).

Using DEAE-Sephadex A-50 at a concentration of 1 mg/ml *van Wezel* demonstrated that a homogeneous microcarriersystem could be used for the large scale culture of anchorage-dependent cells, including diploid human fibroblasts (31). This early work illustrated the potential of the microcarrier technique for producing virus and latter experiments established that this technique could be scaled-up for a variety of large-scale production processes (1,33).

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 (1,32). This toxicity was manifested by the failure of many types of cell to survive the early stages of culture, long lag periods and limited cell yields at the plateau stage of culture.

The explanations for this phenomen have been varied but it is now known that the degree of substitution of DEAE-Sephadex A-50 was not optimal for cell growth (13,14,22,23). 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 (22). Although early experiments on microcarrier culture were not

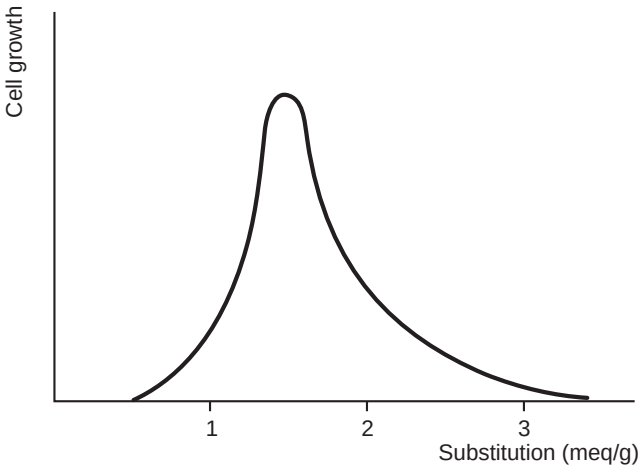


Fig. 4. The relationship between the total degree of DEAE substitution of Sephadex G-50 and growth of cells on the resulting microcarriers. The data were pooled from several studies (16, 22, 23 and unpublished work from Pharmacia Fine Chemicals) and concern growth of several strains of human fibroblasts, 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.

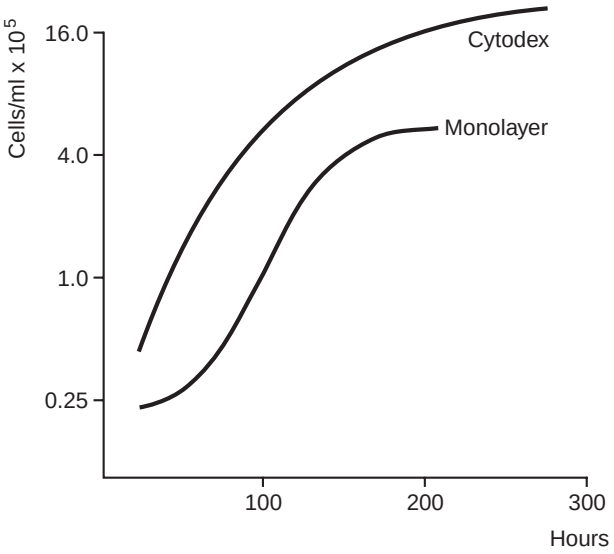


Fig. 5. The growth of primary monkey kidney cells on Cytodex 1 microcarriers and in glass bottles. (Data from van Wezel, A. L., reprodused by kind permission).

controlled or optimized for various culture parameters such as plating efficiency, inoculation density, serum and culture medium, the work of *Kuchler et al* (36), *Inooka* (37) and *Horng and McLimans* (13,14) 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 (16,22,23,33,35).

Figure 4 shows the effect that different degrees of substitution of Sephadex with DEAE-chloride has on cell growth. Using Sephadex as the 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, which was suitable for the growth of a wide variety of cells (17,23,38,39). By using Cytodex 1 the toxic effects associated with DEAE-Sephadex A-50 are avoided and microcarrier concentrations well in excess of 1 mg/ml can be used with concomitant increases in cell yield.

Hence this reduced charge microcarrier was the first product to allow the full potential of microcarrier culture to be exploited at culture volumes up to several hundred litres (10,40,41). Cytodex 1is specifically designed for animal cell culture and satisfies the general requirements for an optimal microcarrier (16,17, section 2.1). When correct culture conditions are used the growth rate of most cells on Cytodex 1 microcarrier is comparable to that achieved on plastic or glass culture surfaces (fig. 5).

The development of Cytodex 1 microcarriers has taken into account the requirements for attachment of cells (section 1.2) and the procedures necessary for maximum growth of a wide variety of cells in microcarrier culture (section 3). The possibilities for microcarrier culture of animal cells have been increased further by the development of Cytodex 2 and Cytodex 3 microcarriers (section 2.3 and 2.4). Since charged groups are necessary only for cell attachment they need only be confined to the surface of the microcarriers.

Cytodex 3 microcarriers represent a new concept in microcarrier culture. Instead of using syntethic charged groups to promote cell attachment, these microcarriers have a surface layer of denatured collagen. Thus the surface upon which cells attach is 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 3.7.2). *Nilsson and Mosbach* (42) have also examined this approach to microcarrier culture.

The relative properties and uses of the various types of Cytodex microcarriers are outlined in section 2.

1.4 Applications of microcarrier culture

Microcarrier culture techniques offer many new possibilities for animal cell culture and the applications fall into three categories, a) high-yield production of cells, viruses or cell products, b) studies on cells *in vitro* and c) routine cell culture techniques. With Cytodex these applications can be realized for a very wide variety of different cells. The applications of microcarrier culture are advancing rapidly and additional information can be obtained from Pharmacia Fine Chemicals.

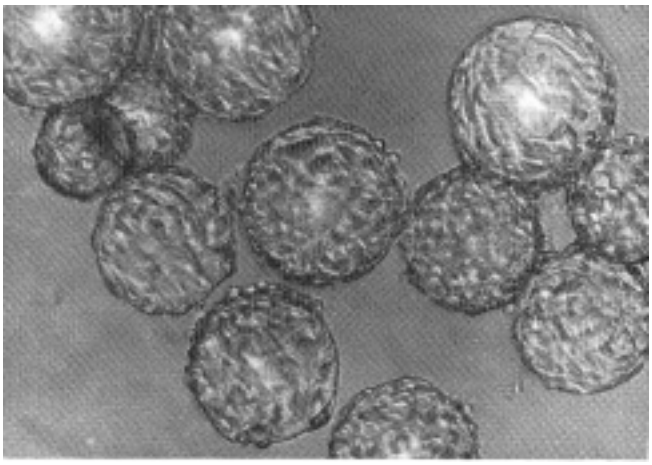


Fig. 6. Human-mouse hybrid cells growing on Cytodex microcarriers. The parental cells for the hybrid were human lymphocytes and HGRT mouse cells. (Original photograph by B. Winchester, Queen Elizabeth Collage, London, UK, reproduced by kind permission).

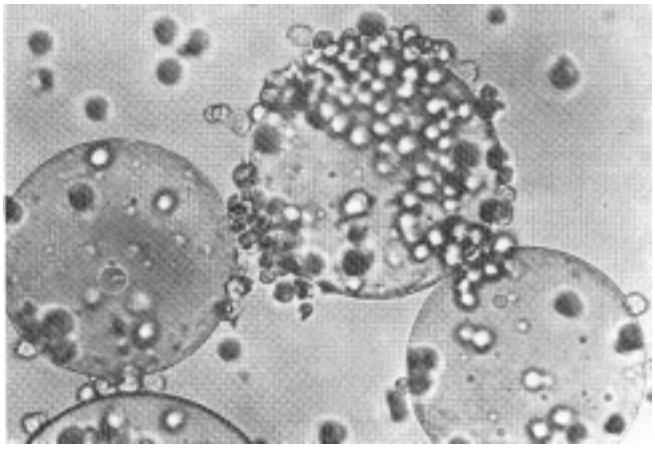


Fig. 7. Hybrid of mouse spleen cells and human bladder tumour cells growing on Cytodex microcarriers.
(Original photograph by A. O'Toole, London Hospital Res. Lab., London, UK, reproduced by kind permission).

1.4.1 Cell types cultured on Cytodex microcarriers

The cell types successfully cultured on microcarriers are listed in Section 6.1. and examples of virtually all classes of cultured animal cells are represented. Cytodex microcarriers are cell culture surfaces of general applicability and provided a cell is capable of attachment *in vitro* it will be able to attach to the microcarriers. Cytodex 3 also provides an improved culture surface for many types of cells which attach or function poorly on glass or plastic culture surfaces (section 2.4).

Mammalian, avian, fish and insect cells have been cultured on Cytodex. These cells are of wide histotypic origin and include primary cells, diploid cell strains and established or transformed cell lines. Hybrid cell lines and cells of tumour origin can be cultured on Cytodex microcarriers (figs. 6,7,8). Selection of the most suitable microcarrier for these cells depends on the cell type and the application (section 2.5). Examples of cells growing on Cytodex microcarriers are shown in Plates 1-9 and other figures throughout this book.

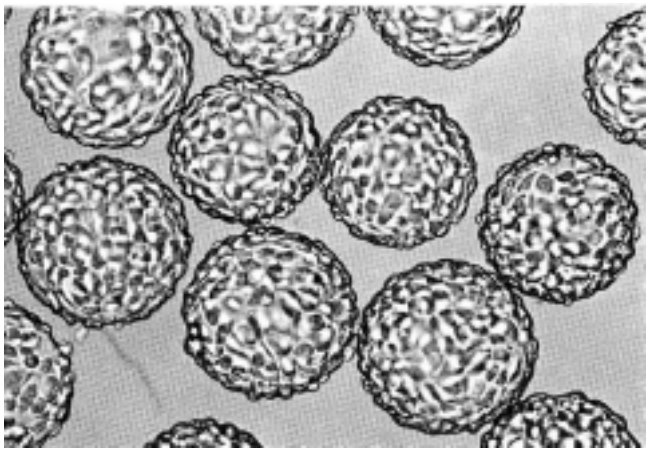


Fig. 8. Human osteosarcoma cells cultured on Cytodex microcarriers.
(Original photograph by B. Westermark and J. Pontén. Wallenberg Lab., Uppsala, Sweden, reproduced by kind permission).

The only types of cells which have proveded to be difficult to grow on microcarriers in stirred cultures have been some of lymphoid origin. Such cells attach only weakly to cell culture surfaces and can be dislodged from the microcarriers if the culture is stirred too vigorously. Lymphocytes and lymphoblastoid cells have been successfully cultured on Cytodex microcarriers (43, plate 4, *G Alm*, pers. comm., 184) and good attachment can be achieved when correct procedures are used (section 3.4,3.5). Cytodex is not mitogenic in cultures of lymphocytes (43).

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 while still retaining the benefits of suspension culture techniques.

- Higher culture densities can often be achieved with microcarriers and productivity of the culture can be increased.
- Separation of the cells from the liquid phase of the culture is more simple when using microcarriers - long sedimentation times, complicated filters or centrifuges are not required when harvesting the cells.
- 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 be therefore be enriched for living cells.

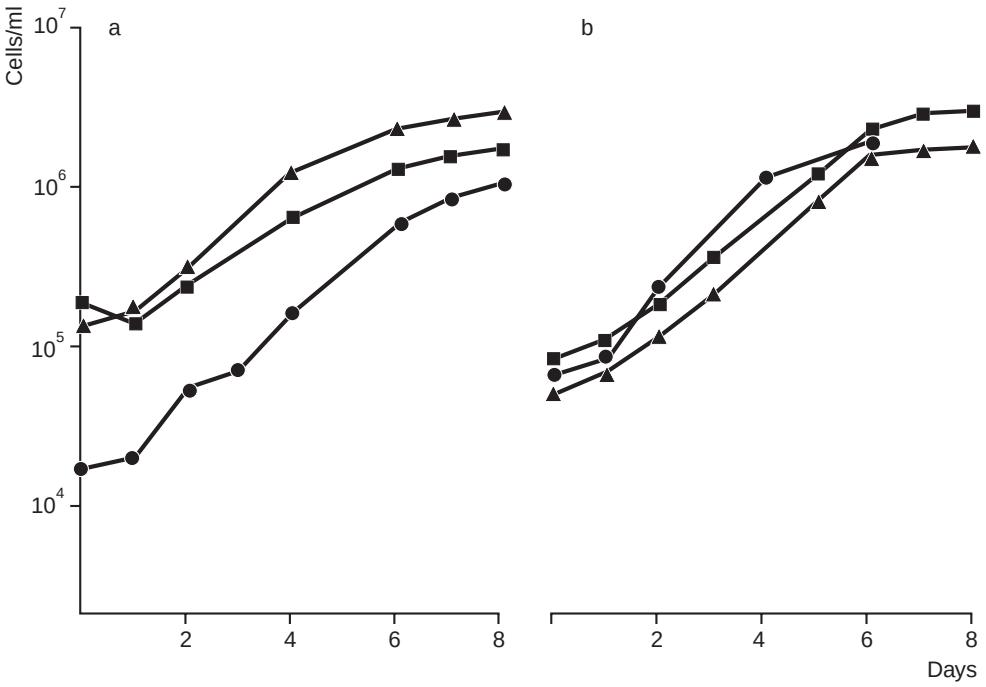


Fig. 9. The growth of various types of cells on Cytodex microcarriers in stirred cultures.
a. Human fibroblasts (MRC-5, —■—), chicken fibroplasts (—▲—), human nasophararangeal carcinoma cells (KB, —●—; data reproduced by kind permission of S.Toyama, Inst. Virus Res., Kyoto University, Kyoto, Japan).
a. Mouse fibroblasts (J-129, —■—), normal rat kidney cells (NRK, —▲—), rhesus monkey kidnney cells (LLC-MK_2, —●—; data reproduced by kind permission of S.Toyama, Inst. Virus Res., Kyoto University, Kyoto, Japan).

- Cells growing on culture surfaces often use medium more efficiently than the same cells growing in free suspension (44). Microcarriers provide a method for reducing 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 culture systems.

In general cells have the same growth kinetics on Cytodex microcarriers as they do on standard glass or plastic culture surfaces (figs. 5,25). Provide culture conditions are optimal (sections 3.4,3.5) most cells will retain their characteristic morphology, population doubling time and saturation density when growing on Cytodex microcarriers (figs. 5,9). Cells which 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.

1.4.2 Production of large numbers of cells

A major area of application for microcarrier culture is the production o 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.9). This high yield of cells per unit culture volume and the large increase in cell number during the culture cycle (10-fold or more) make microcarrier culture an attractive technique for production of cells from a wide range of culture volumes.

Applications for small culture volumes include situations when only 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

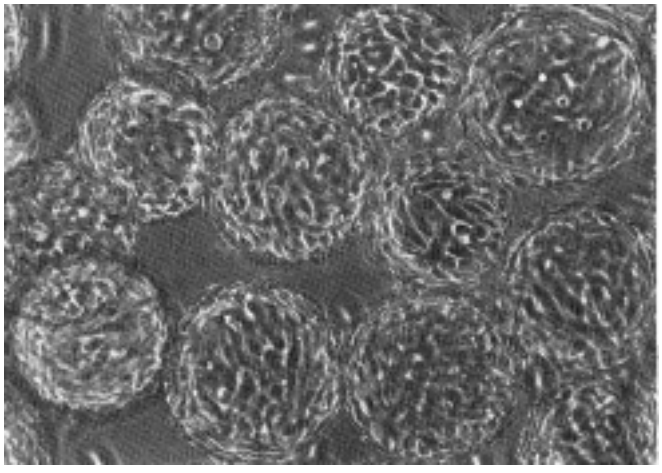


Fig.10. Chinese hamster fibroblasts growing on Cytodex microcarriers contained as a static culture in a Petri dish. (Original photograph by T. Utakoji, Cancer Inst., Japanese Fondation for Cancer Res., Tokyo, Japan, reproduced by kind permission).

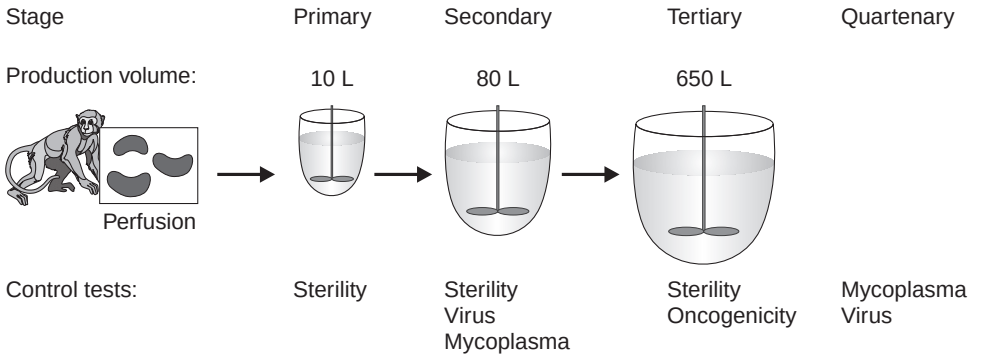


Fig.11. Scaling up microcarriers cultures for the production of large numbers of cells. Example illustrates the subcultivation system for production of polio vaccine from monkey *(Cynomolgus)* kidney cells growing on Cytodex microcarriers. (Adapted from van Wezel, A.L., van der Velden-de Groot, C.A.M., van Herwaarden, J.A.M., *Develop. Biol. Standard. 46 (1981) 151*).

a high culture surface area/volume ratio (approx. 4 cm^2/ml in Petri dishes). Microcarrier cultures provide a surface area/volume ratio of approx. 20 cm^2/ml. The increase in culture surface area means that a greater yield of cells is achieved before subculturing is necessary. In the area of clinical diagnosis or production of cloned material this technique leads to a reduction in the time required to grow cells for biochemical analyses (45).

Figure 10 illustrates the high yield of cells which can be obtained from microcarrier cultures contained in traditional monolayer culture vessels. Microcarrier culture also provides a method for rapid scaling-up with a minimum number subculture steps (fig. 11). Scaling-up can be through the entire range of culture volumes and can also be achieved in the absence of subculturing steps by using a continuos propagation technique (6). This technique provides sustained periods of exponential growth. Large culture volumes of several litres or more are mainly used for production of viruses or cell products (section 1.4.3). The yield of cells from large-scale cultures using Cydotex is usually 10^9 cells/litre or more.

1.4.3 Production of viruses and cell products

Cells cultured on microcarriers are often used as substrates for the production of viruses or cell products and the microcarrier method is compatible with standard production procedures. Cytodex can be used for the production of all substances which 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 (Table 1). 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 (1,40,46,48). Vaccines produced in the microcarrier system include polio, rubella, rabies, influenza, and foot-and-mouth disease (FMD) vaccines (1,10,40,41,46,48-50). Figures 12 and 13 illustrate the growth of Vero cells and the production of Herpes simplex virus on microcarriers.

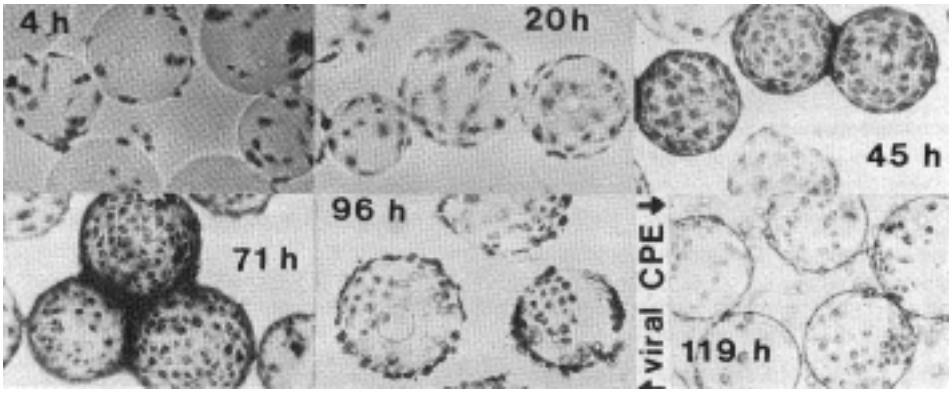


Fig.12. Culture of Vero cells on Cytodex microcarriers used for the production of *Herpes simplex* virus (HSV-2). The culture was infected with HSV-2 after approx. 50 h. CPE-cytopathic effect. (Original photograph by B. Griffiths, CAMR, Porton Down, UK, reproduced by kind permission).

Fig.13. The growth of Vero cells and *Herpes simplex* virus (HSV-2 in stirred cultures containing Cytodex microcarriers. Fig. 12. shows photomicrographs of this culture. (Griffiths, B., Thornton, B., McEntee, I., *Eur. J. Cell Biol. 2 (1980) 606*, reproduced by kind permission).

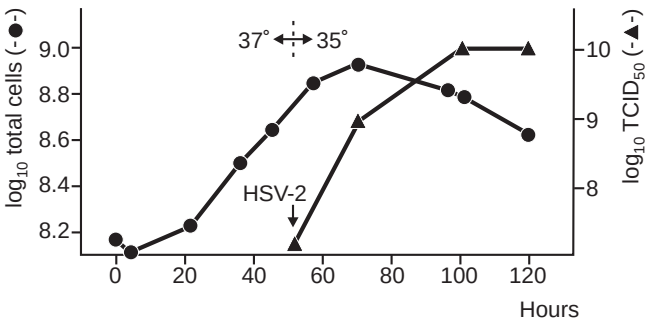


Table 1. Some viruses which have been grown in cultures using Cytodex microcarriers.

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

The advantages of using microcarrier culture for vaccine production include increased productivity, reduced costs and reduced contamination when compared with other cell culture methods (section 1.1). *Sinskey et al* (51) observed that the volumetric productivity of Sindbis virus in microcarrier cultures is in excess of 50-fold greater than that of roller bottles. V*an Wezel et al* (49,59) have developed a "Unit Process" for the production of polio and rabies vaccines using Cytodex microcarriers and the productivity and efficiency of such a system is illustrated in Table 2. An example of a cell culture scheme for the production of inactivated polio vaccine is shown in Figure 11. Serial cultivation on Cytodex reduces the requirement for a source of primary cells and provides a production culture of 650 litres.

Table 2. 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, 40mg/ml; after ion exchange, 8 µg/ml.

Gel filtration was 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 (1979) 65*, by kind permission of the authors and the publisher.).

Table 3. The average yields of polio virus from large scale cultures using Cytodex.

Vero cells[a]: $1.2x10^6$ cells/ml with 1 g Cytodex 1/litre.

Polio virus:	Type I	Type II	Type III
D-Antigen (DU/ml)	85	20	56
Infectivity (log_{10} $TCID_{50}$)	8,1	8,2	7,5

Monkey kidney cells[b]: 10^6 cells/ml with 1-2 g Cytodex 1/litre.

Polio virus	Type I	Type II	Type III
D-Antigen (DU/ml)	80	30	40
Infectivity (log_{10} $TCID_{50}$)	>8	>8	>8

[a] Montagnon, B., Fanget, B.. Nicolas, A.J., *Devel. Biol. Standard. 47 (1981) 55.*
[b] van Wezel, A.L., van Steenis, G., Hannik, Ch.A. et al, *Devel. Biol. Standard. 44 (1978) 159.*

Von Seefried and Chun (46) 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 litres (41).

Polio virus production can also be taken as an example illustrating yields of virus from microcarrier cultures. The yield of polio virus from cultures using Cytodex is 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* (52) observed that the yield of polio virus/cell was greater from microcarrier cultures than from culture flasks.

Cytodex has been used for the production of rabies vaccine by multiple harvests from primary dog kidney cell cultures (48,50). The infective titre of the harvests from these cultures was 6.0±1.0 $\log_{10}$ LD_{50}/ml in mice.

FMD vaccines have been produced from pig kidney cells growing on Cytodex and the vaccines were of good quality with long storage life (10). The FMD vaccines gave good protection of animals with no abnormal local reactions and it was not necessary to concentrate the antigen (10). *Spier and Whiteside* (53) have compared the production of FMD virus from BHK cells grown on microcarriers and in suspension. Microcarrier culture of FMD virus Type 0 gives 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 5 times that obtained from suspension cultures (53).

A large-scale controlled fermenter and Cytodex have been used for the prolonged culture of cells persistently infected with papova virus (54). *Manousos et al* (55) studied the production of oncornavirus in long-term microcarrier cultures and noted that an advantage of this technique was that addition of new microcarriers to confluent cultures caused a new wave of cell growth an virus production. The production of other types of viruses in microcarrier culture is described in references 2,51,56.

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 3-fold greater than production of virus from culture flasks (16.0 $TCID_{50}$/cell).

Interferon has been produced in high yield from microcarrier cultures. The first report (57) described yields of $4x10^3$ IU HuIFNß/10^6 human fibroblasts. A more detailed study examined various parameters and yield were increased to levels comparable to those obtained from traditional monolayer systems (58). *Clark and Hirtenstein* (59) optimized culture procedures for cell growth and modified the induction procedure to result in yields of $3x10^4$ IU HuIFNß/10^6 human fibroblasts. This yield correspondend to $2x10^4$ IU HuIFNß/mg of Cytodex and the technique could be used to produce $3x10^8$ IU HuIFNß/5 litre culture. A procedure for producing HuIFNß is included in section 6.2.1. By using Cytodex microcarriers in roller bottles *Kronenberg* obtained improved yields (approx. 8-fold) of mouse fibroblast interferon (*L. Kronenberg*, pers. comm., 185) The cultures used for these experiments are illustrated in Figure 22. Cytodex has also been used for the production of immune interferon, HuIFNγ (*G. Alm*, pers. comm., 184)

Microcarrier culture has enabled the growth of large numbers of human colon carcinoma cells for the production of carcinoembryonic antigen (60) and the production of plasminogen activator from transformed mouse fibroblasts (*K. Danø*, pers. comm., 186).

Further information on the production and purification of specific viruses and cell products from microcarrier cultures can be obtained from Pharmacia Biotech.

1.4.4 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 the cells. When compared with traditional monolayer techniques which only provide for two-dimensional cultures, the microcarrier system allows for 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 different studies have been reported. The choice of the most suitable microcarrier is described in section 2.5 and for most studies with differentiating systems Cytodex 3 is the microcarrier of choice (table 5). Several examples serve to illustrate the use of microcarriers in cell biology studies.

Pawlowski et al (61) used Cytodex to study the differentiation of chick embryo skeletal muscle cells. Normal myogenesis occured on the microcarriers which were also used for microscopy studies (plate 1). After 4 days of culture 62% of the microcarriers had myotubes with extensive myofibril formation (61).

With an even more sensitive cell system *Moser and Stoffels* (62) 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 20-30% of confluent monolayers on the microcarriers exhibited spontaneous beating activity (62).

The release of insulin from foetal rat pancreas islet cells growing on Cytodex has been studied by *Bone et al* (63,64). These studies demonstrated that Cytodex is suitable for maintaining highly specialized endocrine cells in culture. The

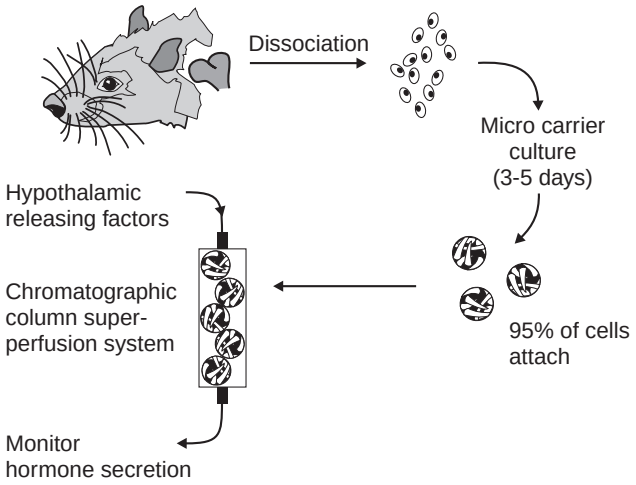


Fig. 14. The superperfusion culture of primary rat pituitary cells growing on Cydodex 1 microcarriers.
The scheme is based on studies by *Smith and Vale* (65, 66).

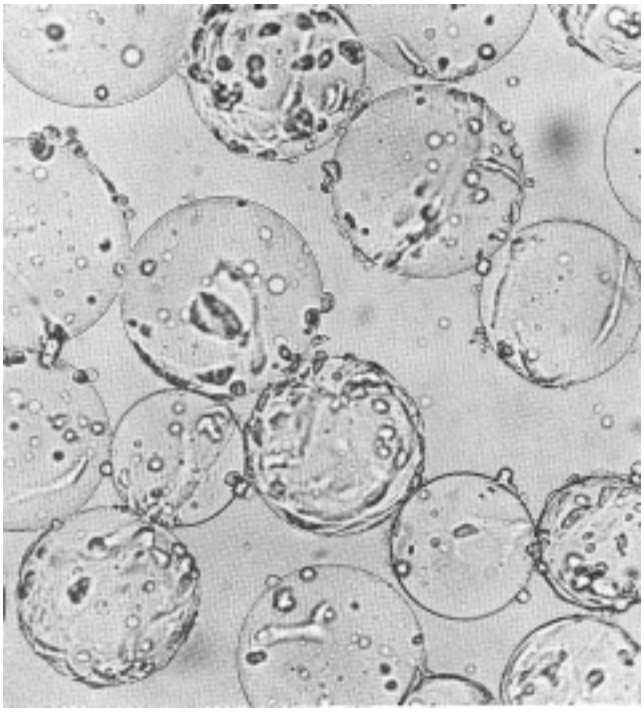


Fig. 15. Primary rat interior pituitary cells attached to Cydodex 1 microcarriers and incubated for 5 days after dissociation. (Smith, M.A. and Vale, W.W. *Endocrinol. 107 (1980) 1425*, by kind permission of the authors and publisher.)

microcarriers provided a method for the uniform suspension culture of functioning pancreas cells and allowed for easy manipulation of the cells (64). The pancreas cells sustained synthesis and release of insulin during a 7 day growth period on the microcarriers (plate 3) and the release could be modulated by glucose and stimulated with theophylline (64).

Microcarriers have been used in novel culture systems to study the function of differentiated cells. *Smith and Vale* (65,66) have developed a superperfusion column technique for the study of rat anterior pituitary cells and the modulation of pituitary secretions by gonadotrophins and cocarcinogens (fig. 14). The system provided responsive and well-defined high density cultures which maintained the ability to secrete hormones fore long periods of time. The dissociated pituitary cells attached to the Cytodex microcarriers (fig. 15) and remained responsive to hypothalamic releasing factors (65,66, fig. 16). Approximately 95% of the cells attached and the culture system could be used to study transient phenomena and desensitization (65).

A variety of other differentiated cells have been studied using Cytodex microcarriers. *Ryan et al* (67) developed a microcarrier culture system for studying the role of bovine pulmonary endothelial cells and *C. Busch* (pers. comm., 187) has used Cytodex 3 in studies of endothelial cells from brain capillary and pulmonary artery (plate 2). Porcine thyroid cells cultured on Cytodex exhibited an epithelial morphology and were capable of releasing thyroglobulin (68).

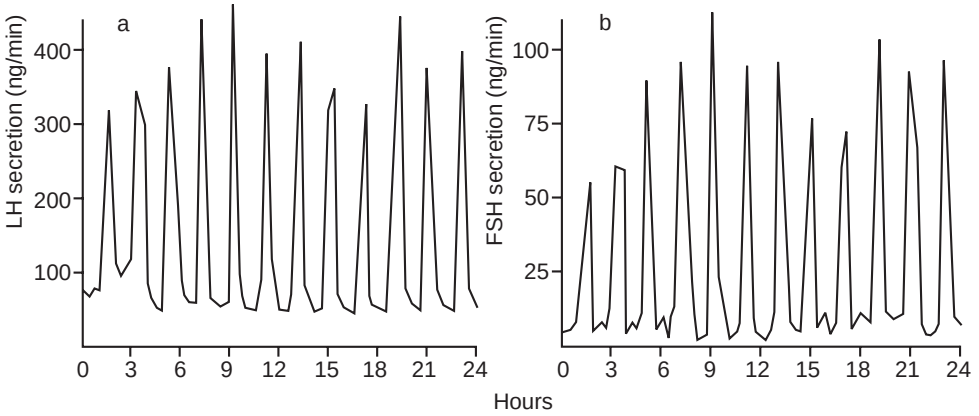


Fig. 16. Response of rat anterior pituitary cells growing on Cydodex 1 microcarriers to pulses of gonadotropin-releasing hormone (GnRH). The cells were cultured in the system illustrated in fig. 14 and were exposed to 15 min pulses of 30 nM GnRH every 2 h. Flow rate was 0.2 ml/min and fractions were collected every 20 min.
a. Secretion of lutenizing hormone.
b. Secretion of follicle stimulating hormone.
(Smith, M.A. and Vale, W.W., *Endocrinol. 108 (1981) 752,* by kind permission of the authors and publisher.)

Vosbeck and Roth (69) used microcarrier culture to study the effects of different treatments on intercellular adhesion. Confluent monolayers of cells were cultured on microcarriers and intercellular adhesion was examined by studying the binding of ^{32}P-labelled cells to the monolayers (69).

Lymphocytes have been grown on microcarriers for studies of stimulation (43). Cytodex 1 alone was not mitogenic for lymphocytes but potentiated stimulation by Con A (fig. 17). The microcarriers have been used to study the relationship between anchorage, cell density and stimulation of lymphocytes (43).

Microcarriers are also used in studies of animal cell plasma membranes. *Lai et al* (70) used Cytodex to study the influence of adhesion on the fluidity on Chinese hamster ovary cell plasma membranes. By using electron spin resonance technique it was possible to compare cells growing in free suspension culture and attached to microcarriers in suspension culture. Cytodex microcarriers are compatible with spin-labelling and provided a technique whereby cells could be easily transferred and assayed without removal from the culture surface (70).

Microcarrier culture can also be used for the isolation of plasma membranes with less than 1% contamination from internal membrane markers (71). 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 (71).

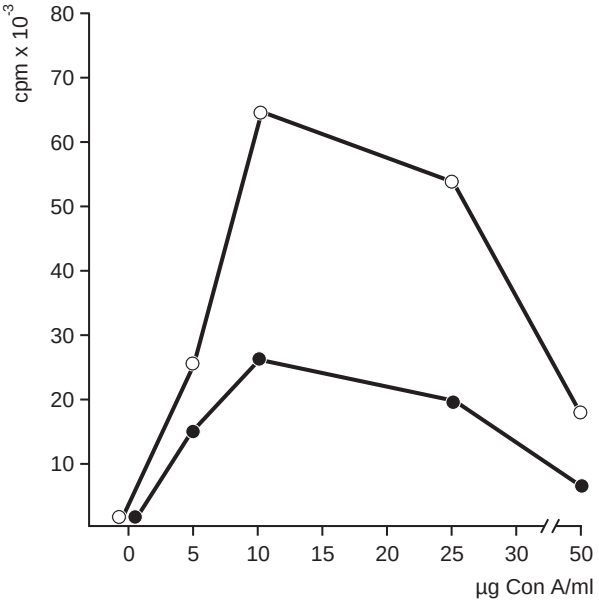


Fig. 17. The stimulation of human lymphocytes by Con A in the presence (❍) or absence (●) of Cydodex microcarriers. The microcarriers alone were not mitogenic. (Sundqvist, K. and Wagner, L., *Immunology 43 (1981) 573*, by kind permission of the authors and the publisher.)

1.4.5 Proteolytic enzyme-free subcultivation and cell transfer

In many studies it is important to be able to harvest or transfer cultured cells without using proteollytic enzymes or chelating agents. Such agents often alter cell viability and the integrity of the plasma membranes. By using microcarriers it is possible to subculture cells or scale-up cultures without using proteolytic enzymes or chelating agents. Microcarriers 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 (72) and R*yan et al* (67) observed that cells can migrate between microcarriers and the surfaces of cell culture flasks or Petri dishes. Cytodex microcarriers were allowed to settle onto monolayers of mouse fibroblasts and cells migrated onto the microcarriers which could then be transformed to another culture vessel (72). This method of transfering cells has also been used for bovine pulmonary artery endothelial cells; such cells are normally sensitive to treatment with proteolytic enzymes (67).

A variant of this technique is to allow cells to migrate from confluent microcarriers onto new microcarriers. *Crepsi andt Thilly* (6) could maintain prolonged periods of exponential growth of CHO cells by diluting confluent microcarrier cultures and adding new microcarriers. The transfer of cells between microcarriers was enhanced by using a medium with low Ca^{2+} concentration (6). New microcarriers can also be added to confluent microcarrier cultures during periods of virus production and *Manousos et al* (55) 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 and after a few hours, migration of cells results in inoculation of the new microcarriers (*P. Talbot*, pers. comm., 188)

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, Cytodex can be incubated with peritoneal fluid and after 10 min macrophages adhere to the microcarriers and can be separated from the other peritoneal cells by simple differential sedimentation (*H. Slater,* pers. comm., 189). The macrophages attached to the microcarriers can then be transfered 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 transfered by Pasteur pipette into cloning wells. In this way the microcarriers provide an easily manipulated culture surface. Similarly confluent microcarriers can be embedded in semi-solid medium to form feeder layers.

1.4.6 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. 1, plates 1,4,8), transmission electron microscopy (fig. 3) and different types of light microscopy illumination and cytochemistry (plates 2,3,5-7,9). The advantages of using microcarriers for microscope is that such 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. 3)

Routine samples from microcarrier cultures can be processed for detailed microscopical examination and cultures containing many coverslips can be 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 3.6.

1.4.7 Harvesting mitotic cells

Microcarrier culture provides an efficient method for harvesting mitotic cells (73,74). The technique is based on the observation that mitotic cells are attached only weakly to cell culture surfaces and can be detached by shaking (75). The use of monolayer culture vessels for this technique is limited by the small surface area for cell growth and 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* (73) reported that CHO cells harvested from Cytodex microcarriers by this method had a mitotic index of up to 88%, a considerable improvement over the mitotic index of 41% obtained when harvesting mitotic cells by shaking from a culture flask.

Ng et al (74) 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 $4x10^4$ mitotic cells/ml of microcarrier culture could be obtained. These cells had a mitotic index of 85-95% (74).

1.4.8 Transportation and storage of cells

The large surface area/volume ratio of microcarriers is advantageous when transporting and storing culturing cells. Large numbers of cells (up to approx. 10^7 cells/ml) can be transported or stored whilst still attached to the culture substrate. This technique avoids transportation of large numbers of monolayer culture vessels (e.g. flasks) and also eliminates the need to store or transport suspensions of anchorage-dependent cells. The advantage of transporting or storing cells attached to a culture surface rather than a suspension is that loss of cells associated with harvesting and replating is avoided. After transportation or thawing from storage the cells are already attached to the culture surface and can continue to function and proliferate.

Procedures for storing most anchorage-dependent cells whilst still attached to Cytodex microcarriers are described in section 4.6. and even cultured insect cells can be frozen and stored in liquid nitrogen when attached to microcarriers (*E. Duda,* pers. comm., 190)

2. Cytodex microcarriers

2.1 Requirements for an optimum microcarrier

In order for a microcarrier to be suitable for animal cell culture at all scales it must fulfill certain basic criteria (16).

- **Surface properties** must be such that cells can adhere with a degree of spreading which permits proliferation. For homogeneous growth of cells the surface of the microcarrier must have an even, continuos contour. The surfaces of all microcarriers in the culture should have consistent properties.
- **Density** of the microcarriers should be slightly greater than that of the surrounding medium, thus facilitating easy separation of cells and medium. The density should also be sufficiently low to allow complete suspension of the microcarriers with only gentle stirring. Under standard culture conditions the optimum density for microcarriers is 1.030-1.045 g/ml.
- **Size** distribution should be narrow so that even suspension of all microcarriers is achieved and that confluence is reached at approximately the same stage on each microcarrier. Best growth of cells occurs when microcarriers have a size distribution which lies within limits of diameter in culture of 100-230 µm.
- **Optical properties** should be such that routine observation of cells on microcarriers can be achieved using standard microscopy techniques. The microcarriers should also permit use of routine cytology procedures.
- **Non-toxic** microcarriers are required not only for survival and good growth of the cells but also when cell culture products are used for veterinary or clinical purposes.
- **Non-rigid** microcarrier matrices are required for good cell growth when the culture is stirred. Collisions between microcarriers occur in stirred cultures and a compressible matrix reduces the possibility of damage to the microcarriers and the cells.

All Cytodex microcarriers are designed to meet the above requirements and are based on a spherical matrix of cross-linked dextran. This matrix was chosen because it provides a microcarrier with suitable physical properties and also because dextran in non-toxic, having widespread use in clinical applications and for the preparation of pharmaceutically important materials (see ref. 76)

Cytodex microcarriers are non-toxic and provide surfaces which can be used for the cultivation of a wide variety of cell types (section 6.1). These microcarriers have a size distribution and density compatible with the culture procedures required for optimal cell growth. Furthermore Cytodex microcarriers are sufficiently strong to withstand normal culture procedures and conditions but are non-rigid and have excellent optical properties.

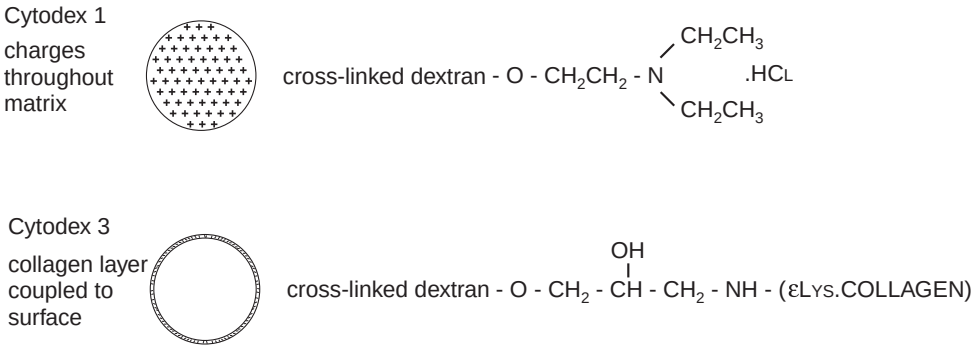


Fig. 18. Schematic representation of the three alternative types of Cydodex microcarriers.

2.2 Cytodex 1

Cytodex 1 microcarriers are based on a cross-linked dextran matrix which is substituted with positively charged N,N-diethylaminoethyl (DEAE) groups to a degree which is optimal for cell growth (fig. 4). The charged groups are found throughout the entire matrix of the microcarrier (fig. 18).

Published procedures for substituting cross-linked dextran with DEAE groups to form microcarriers for cell culture (3,22,35,55) can lead to the formation of a high proportion (up to 35%) of tandem charged groups (see 24). 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); thus stability and homogeneity of the charged groups is enhanced and possible leakage of charged groups from the microcarriers is minimized. *Windig et al* (77) 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 (77).

The physical characteristics of Cytodex 1 are summarized in Table 4.

2.3 Cytodex 3

Cytodex 3* microcarriers are based on an entirely different principle for microcarrier culture. While most surfaces used in cell culture posses a specific density of small charged molecules to promote attachment and growth of cells (e.g. glass, plastic, Cytodex 1,), certain proteins can also provide a surface for the growth

*patents pending

of cells in culture. The connective tissue protein, collagen, has proved to be a valuable cell culture substrate. Cytodex 3 microcarriers consist of a surface layer of denatured collagen covalently bound to a matrix of cross-linked dextran (fig. 18). The amount of denatured collagen bound to the microcarrier matrix is approx. 60 μg/cm^2 and results in maximum cell yields (24). The denatured collagen (MW 60,000-200,000) is derived from pig skin type I collagen which has been extracted and denatured by acid treatment, concentrated and purified by an ion exchange step and steam sterilized before being coupled to the microcarrier matrix. These microcarriers combine the advantages of collagen coated culture surfaces (see below) with the advantages and possibilities of microcarrier culture (section 1.1, 1.4). Cytodex 3 microcarriers 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 cell culture surfaces. Consequently, collagen-coated culture surfaces are used frequently for establishing primary cultures and for growing cells which are normally difficult to grow in culture (78, 79). Collagen-coated surfaces are valuable because they permit differentiation of cells *in vitro* even at very sparse or colonel culture densities (79,80). 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 (81). 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 (82). Differentiation of myoblasts at sparse densities *in vitro* depends on the presence of collagen bound to the culture surface (80, 81). Myoblasts attach and spread more satisfactorily on collagen than on standard cell culture surfaces (81) and growth is also stimulated (83).

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 (84). Since exogenous fibronectin is not required for attachment of hepatocytes to collagen (85) this culture surface is the most suitable surface for culture of hepatocytes in protein-free media (84). A collagen culture substrate also allows for more extended studies of hepatocytes *in vitro* with improved retention of differentiated function (86,87).

Folkman et al (88) 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 (plate 2). *Geppart et al* (89) 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 for the culture of fibroblasts (90, 91) and *Ceriejido et al* (92) described the use of cross-linked collagen for the culture of 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. 19).

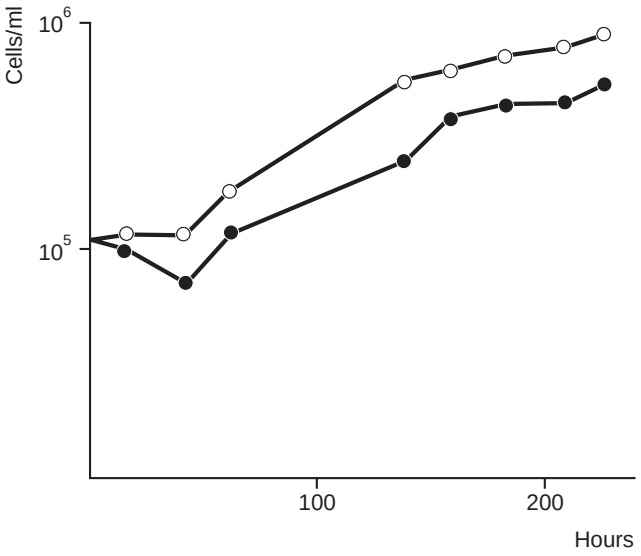


Fig. 19. The growth of secondary bovine embryo kidney cells on Cytodex 1 (—●—) and Cytodex 3 (—○—) microcarriers. The cells were a mixed population with predominantly epithelial morphology.
(Gebb, Ch., Clark, J.M., Hirtenstein, M.D. et al, *Develop. Biol. Standard. (1981)*, in press, by kind permission of the authors and the publisher).

Cytodex 3 microcarriers are coated with denatured Type I collagen because this is the most generally useful form of collagen for cell culture . Although certain types of cells may show a specificity for attachment to a particular form o native collagen, this specificity 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 1.2) and fibronectin is believed to be also involved in the attachment of many cells to both native and denatured forms of collagen (93). Fibronectin binds equally well to all types of collagen (94) but shows a preference for denatured forms (82,95,96) and binds more rapidly to Sephadex® beads coated with denatured collagen than to beads coated with native collagen (97).

Table 4. 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)	4,400	2,700
Approx. no microcarriers /g dry weight	$6.8x10^6$	$4.0x10^6$
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.

Table 5. Selection of Cytodex microcarrier based on cell type and application area. The choice is also modified under certain conditions (section 2.5).

	Cell type[a]									
Application area[b]	*Primary*[c]	fibroplast	epithelial	mixed	tumour	*Normal diploid cell strains*	*Established cell lines*	fibroplast	eqithelial	
Cultures for production										
General purpose cell production										
Production of virus		1	1,3	1,3	1,3	1[d]		1	1,3[e]	
Production of cell products										
Cultures for other cell biology studies										
Differentiated culture systems[f]					Cytodex 3					
Microscopy		1	1,3	1,3	1,3	1		1	1,3	
Uptake studies		3	3	3	3	3		3	3	
Isolating cells with membranes intact					Cytodex 3					
Harvesting mitotic cells		1	1,3	1,3	1,3	1		1	1,3	

[a] Definition of cell type is based on morphology in culture. Cells of epithelial, endothelial and mesothelial origin usually have an epithelial morphology in culture.
[b] More details on applications can be found in section 1.4.
[c] Also includes secondary, tertiary etc. cultures which have not become established.
[d] Cytodex 3 can be used for high yields of certain cell strains, e.g. FS-4.
[e] Cytodex 3 is more suitable than Cytodex 1 for cell lines with pronounced epithelial morphology e.g. BSC-1.
[f] For example hepatocytes, hepatomas, muscle cells, endocrine cells etc.

In the past, a major difficulty with using collagen coated surfaces has been the rapid loss of collagen from the surface. *Kleinman et al* (93) observed that up to 40% of the collagen is lost from coated Petri dishes within 90 minutes of adding culture medium. Floating collagen gels (79) have greater retention of collagen but are difficult to prepare and often shrink during the culture period (87). Because the denatured collagen is cross-linked to the microcarrier matrix, the problems of shrinkage, cracking and leakage of collagen which are found with standard techniques for coating cell culture surfaces are avoided with Cytodex 3 microcarriers. A further advantage of using Cytodex 3 microcarriers as a general purpose collagen-coated culture surface is that beause the microcarriers have a porous matrix, nutrients also 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 microcarriers is susceptible to digestion by a variety of proteases, including trypsin and collagenase (section 3.7.2) and provides unique opportunities for removing cells from the microcarriers.

The physical characteristiques of Cytodex 3 are summarized in table 4.

2.4 Which Cytodex microcarrier to use?

Choosing the correct Cytodex microcarrier will depend on the type of cell being cultured and the purpose of the culture (table 5). Cytodex 3 should be used for situations not included in table 5. Certain conditions or culture requirements may modify the choice of microcarrier indicated in table 5. In addition to being a microcarrier Cytodex 3 can be used as a general purpose collagen-coated cell culture substrate.

- **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%) it is important to use the microcarrier which enables attachment of the maximum number of cells. In table 5 there are listed certain situations where more than one type of microcarrier can be used. In some cultures it may be necessary to use the microcarrier which results in the highest plating efficiency. When culturing cells with fibroblast-like morphology, Cytodex 1 is a suitable alternative. For cells having an epithelial-like morphology, plating efficiency is greater with Cytodex 3 than Cytodex 1. Selecting the microcarrier in this way can also result in higher cell yields, simply because the plating efficiency is improved (fig. 19).

- **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 of cells. This situation often arises when working with primary cultures derived from small quantities of tissue. Under such circumstances it is important to use the microcarrier which results in the highest plating efficiency (see above). For primary cultures inoculated with less than approx. 5 viable cells/microcarrier Cytodex 3 is recommended instead of Cytodex 1.
  If only small numbers of cells from established cell lines having a low plating efficiency (i.e. less than approx. 10%) are available for inoculation, then Cytodex 3 i 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, it is advisable to 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 microcarrier culture. Under such conditions Cytodex 3 should also be considered for cells with fibroblasts morphology. The sometimes slower growth of fibroblasts on Cytodex 3 is usually compensated by the improved harvesting and viability of the harvested cells. Hence it is advantageous to use Cytodex 3 for scaling up cultures and for fibroblasts the final production culture can then use Cytodex 1.

The improved harvesting techniques possible with Cytodex 3 are described in section 3.7.

- **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 be able to wash medium components or cell products from the culture. The amount of protein which 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 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 6 illustrates that it is possible to wash out serum proteins to a much greater extent from cultures with Cytodex 3 than from cultures using Cytodex 1. When it is important to remove medium components easily or to achieve maximum recovery of cell products Cytodex 3 are preferred.

Table 6. Removal of serum proteins from cultures using Cytodex microcarriers. (Results kindly supplied by Dr. A.L. ven Wezel).

Sample*	Cytodex 1		Cytodex 3	
	Albumin	IgG	Albumin	IgG
Culture fluid	>1/2,000	>1/125	>1/2,000	>1/125
First wash	>1/2,000	1/500	>1/2,000	1/500
Second wash	>1/2,000	1/4,000	1/32,000	1/16,000
Third wash	1/8,000	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 resupended in Medium 199 without serum in 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 counter-current electrophoresis and a dilution of 1/32,000 corresponded to 1 mg albumin/ml or 1 mg IgG/ml.

2.5 Availability and storage

All Cytodex microcarriers are supplied as a dry powder and must be hydrated and sterilized before use (section 3.3). The following pack sizes are available:

Cytodex 1	25 g	100 g	500 g	2.5 kg	5 kg
Cytodex 3	10 g	100 g	500 g	2.5 kg	5 kg

Packs of Cytodex should be opened and stored under dry conditions.
Stored unopened at room temperature Cytodex microcarriers are stable for more than 8 years.

3. Microcarrier cell culture methods

3.1 General outline of procedure

Microcarrier culture is a versatile technique for growing animal cells and can be used in a variety of different ways for a wide range of applications (section 1.4). 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 references 165,166,175,177.

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 traditional monolayer culture is invaluable when deducing the most suitable microcarrier culture procedure. The best procedure is the one which ensures maximum attachment of the inoculum to the microcarriers and results in rapid, homogeneous growth of cells to the highest possible culture density. The aim of section 3 is to outline the basic principles of microcarrier culture. These priciples provide the background necessary for deducing the best culture procedures for a wide variety of cells without needing to spend time on extensive preliminary experiments. Examples of specific culture procedures are given in section 6.2.

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

Step

1. Choose the most suitable Cytodex microcarrier based on cell type and application (section 2.5).
2. Select the most suitable culture vessel for the application. The best results and highest yields are obtained from microcarriers maintained in suspension (section 3.2), but a static culture is often use in an initial experiment (step 4).
3. Hydrate and sterilize the microcarriers (section 3.3).
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 3.4). A rounded morphology and any tendency of the cells to clump will also define what stirringspeeds should be used (section 3.5). A rapid fall in pH of the culture (the medium turns yellow within 3 days) will indicate that modification to the medium may be necessary for cultures having a high density (section 3.5.2,4.1).
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 3.4 and 3.5 describe the principles of choosing the most suitable conditions.

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 3.4.6,3.5.2,4.1) and to alter the supply of gases (section 4.3). In addition, greater economy of inoculum and serum may be achieved by altering the culture conditions (section 3.4,4.2). More information on optimizing culture conditions and trouble shooting is found in section 5.

3.2 Microcarrier culture vessels

3.2.1 Requirements

In principle microcarrier culture is a very flexible technique and cultures can be contained in virtually any type of cell culture vessel. Microcarriers can be used simply to increase the surface area of static cultures in traditional vessels or can be used in genuine stirred suspension cultures where the full benefits of the microcarrier technique can be realized. In all situations the culture vessel must be non-toxic and sterile.

The best results from microcarrier cultures are obtained when using equipment which gives even suspension of the microcarriers with gentle stirring and possibilities for adequate exchange of gases with the culture medium. Erratic stirring motions should be avoided since these lead to detachment of 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 exposing the culture to vibration. Stirring mechanisms should be checked for vibration. Magnetic stirring units are often a source of vibration and a simple method for reducing transmission of vibration to the culture vessel is to place a thin piece of plastic foam between the culture vessel and the surface of the base unit.

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 approximately 5 liters and can be contained in a wide variety of vessels. Large scale microcarrier cultures range from approximately 5 to several hundred liters and must be maintained in specially designed vessels (fermenters) allowing monitoring and control of parameters such as gas tensions and pH.

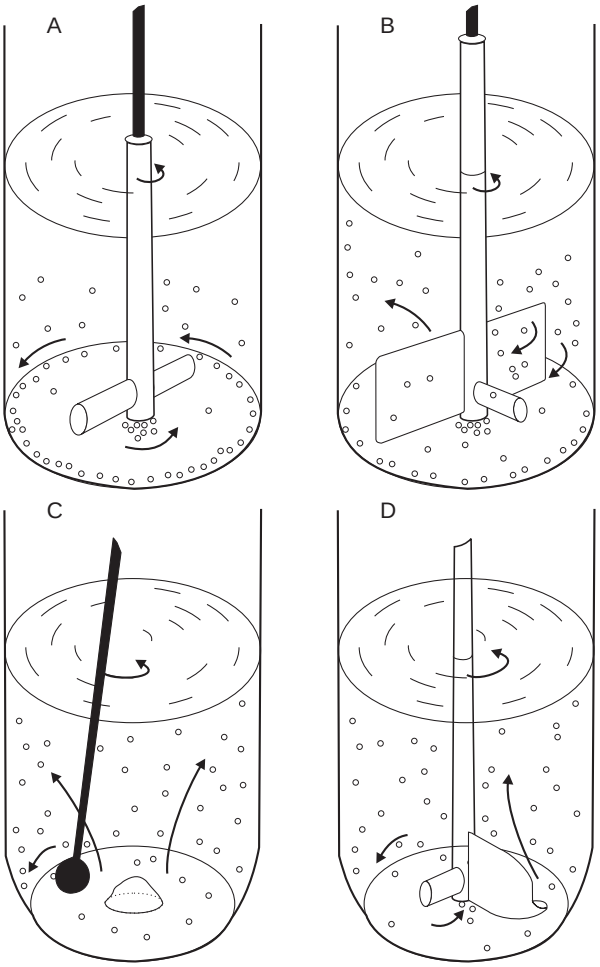


Fig. 20. Various stirred culture systems for laboratory scale microcarriers culture. All systems are stirred by a magnetic base stirring unit.
A. Traditional spinner vessel of type supplied by Bellco Glass Inc. or Wheaton Scientific. Stirring speed 50–60 rpm.
B. Spinner vessel modefied for microcarrier culture: Paddle impeller (Bellco Glass Inc.). Stirring speed 20–40 rpm.
C. Rod-stirred microcarrier culture contained in a vessel with idented base (Techne (Cambridge) Ltd.) A magnet is present in the bulb of the stirring rod. Stirring speed 20–30 rpm.
D. Spinner vessel modefied for microcarrier culture: Plough impeller. Vessel with round bases were supplied by Wheaton Scientific and the plough-shaped impeller was fashioned from Teflon®. Stirring speed 15–30 rpm. (Hirtenstein, M., Clark, J.M., Gebb, Ch., *Develop. Biol. Standard. (1981), in press,* by kind permission of the authors and the publisher).

3.2.2 Laboratory scale microcarrier culture vessels

Spinner and rod-stirred vessels

The most suitable vessels for general purpose laboratory scale microcarrier culture are those having a stirring rod or impeller driven by a magnetic base unit (fig. 20). Cultures stirred by a bulb-shaped rod (fig. 20) produce higher yields of cells than cultures stirred by the spinner principle (fig. 21) 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 which is 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 which is immersed in the culture medium are not suitable, since the microcarriers can circulate through the bearing and become crushed. When using

spinner vessels the position of the impeller should be adjusted so as 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, then an open, monitored culture system is advisable (section 3.2.3). 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 tensions (section 4.3,4.4). 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. 33, section 3.5.2).

While good results with many cell types can be obtained from traditional spinner vessels, recently developed vessels and magnetic stirrers can be obtained from Bellco Glass Inc. (Vineland, NJ, USA) who have modified the traditional spinner vessel for use with microcarriers (fig. 20).

Wheaton Scientific (Milville, NJ, USA) and Wilbur Scientific Inc. (Boston, MA,USA) also supply spinner vessels and magnetic stirring bse units which are suitable for microcarrier culture (22). Culture vessels modified with rounded bases can be supplied on request (fig. 20).

An inexpensive spinner vessel for culture volumes of between 2 and 20 ml can be made from scintillation vials (98) 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 (Cambrighe) Ltd. (Duxford, Cambridge, UK) have 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 having a rounded and indented base (fig. 20). This system provides a more gentle and even circulation of microcarriers and eliminates the sedimentation of microcarriers often observed when using spinner vessels (99). 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. The increased yield is particularly apparent for cells which have a low plating efficiency (fig. 21). Such a system can be used for cultures with volumes ranging from approximately 100 ml to 3 liters.

Further information on equipment for rod-stirred microcarrier cultures can be obtained from Pharmacia Biotech.

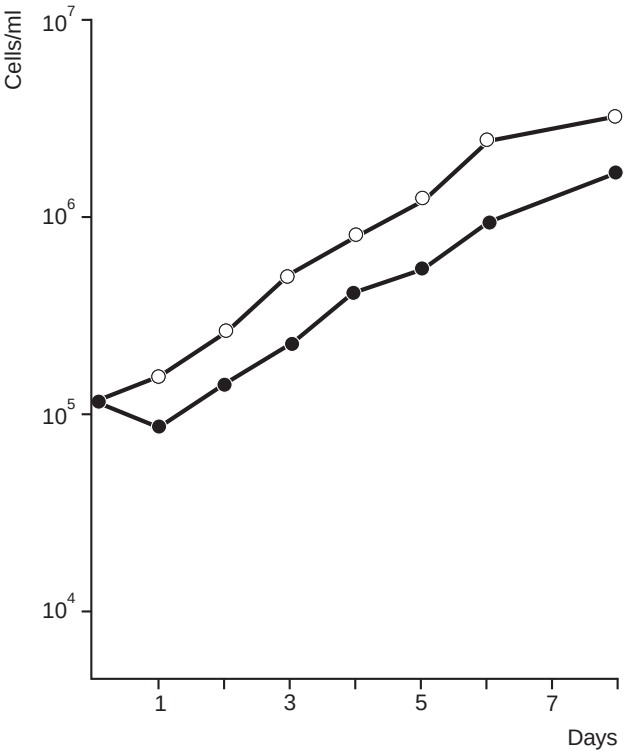


Fig. 21. A comparison of the growth of human fibroblasts (MRC-5) in microcarrier cultures contained in either standard magnetic spinner vessels (—●—) or bulb-shaped rod stirred culture vessel (—❍—). Culture volume was 600 ml and microcarriers were stirred in both systems at a speed just sufficient to achieve even suspension (50–60 rpm, spinner vessel; 20–30 rpm, rod stirred culture). Cultures contained 3 mg Cytodex 1/ml. (From Pharmacia Fine Chemicals AB, Uppsala Sweden).

Roller bottles

Although spinner and rod-stirred cultures provide maximum yields from Cytodex, the microcarriers can 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 from the microcarriers when used under optimal conditions in rod-stirred cultures. Nevertheless by using Cytodex microcarriers it is possible to obtain a 5-10 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 will be dependent on availability of nutrients from the medium, control of pH and an adequate supply of oxygen (see below). There are three alternative methods for using Cytodex with roller bottles.

Method 1: Cytodex can be used to increase the yield from roller bottles by 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. The 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 at given type of cell.

Method 2: The microcarriers can be used for ”suspension” culture in roller bottles. Siliconized roller bottles (section 3.2.4) should be filled 1/2-2/3 full with medium

containing Cytodex at a concentration of not more than 3 mg/ml. The bottles are inoculated with the usual number of cells used for suspension microcarrier cultures (section 3.4.4), flushed with a mixture of 95% air: 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 of the microcarriers. Stringent aseptic techniques are required since the medium comes into contact with the inside of the bottle neck and cap. Modified culture procedures (section 3.4.2) can be used with this method.

Method 3: This method was developed by L. Kronenberg (185, patent pending). Cells are grown to confluence in rotating roller bottles using standard techniques. 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 mins 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 reduction of medium consumption per cm^2 or per 10^6 cells by 50-75% (185). During the microcarrier phase of the culture it is not usually necessary to replenish the medium.

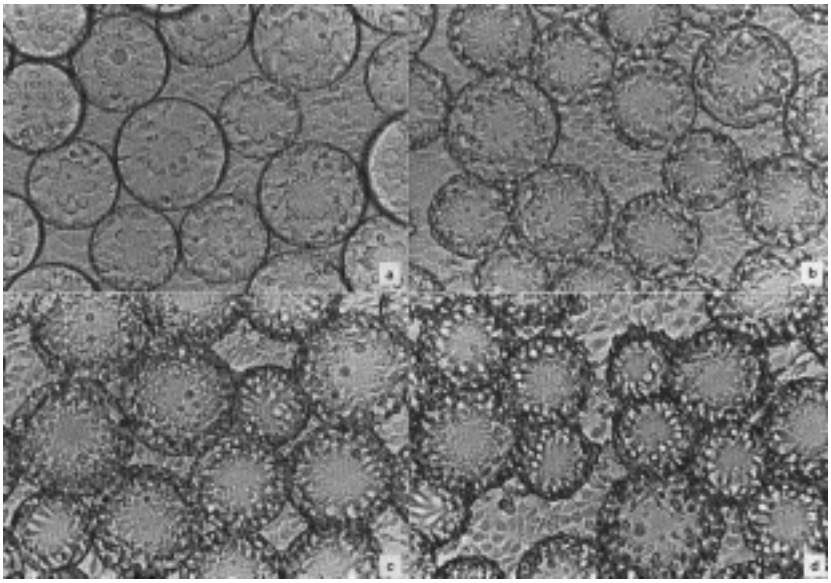


Fig. 22. Roller bottle cultures of mouse L-cells using Cytodex to increase the yield. Confluent cultures were drained and replenished with 400 ml medium containing 1.5 g Cytodex 1 (roller bottles approx. 1400 cm^2). The monolayers were photographed at a) 8 h, b) 24 h, c) 72 h and d) 96 h.
(Original photographs by L. Kronenberg, Lee Bio Molecular Res. Labs. Inc., San Diego, USA, reproduced by kind permission).

The choice of method depends on the circumstances and cell type. Method 2 requires rolling machinery capable of higher speeds of rotation and yields using this method for certain types of cells may be limited by 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. Method 3 is the procedure of choice since the techniques are more simple and reliable than the other two methods. The microcarriers are also fixed or immobilized (fig. 22) and this means that such cultures are compatible with the usual virus/interferon harvesting schemes used with roller bottles and Method 3 has been used to increase the yield of interferon from roller bottle culture (*L. Kronenberg*, pers. comm., 185)

Rocking bottles

The yield of cells from culture bottles (e.g. Roux bottles) can be increased several-fold by using Cytodex. 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) Ficollâ 400 (*S. Smit*, pers. comm., 191) The Ficoll 400 increases the density of the culture medium and thus allows the microcarriers to float more easily. The procedures outlined in section 3.4 are applicable to using this culture method. The rate of rocking should be just sufficient to ensure slow movement of the microcarriers.

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 though the culture . The success of air-lift microcarrier culture depends on using gas of high purity an on defining the gas requirements of

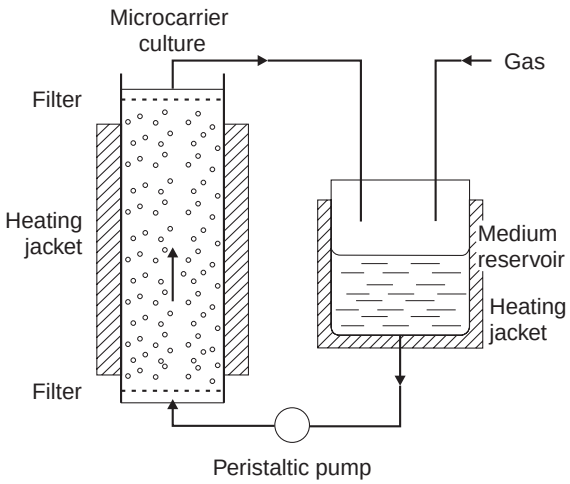


Fig. 23. Schematic diagram of fluid lift microcarrier culture system. The filters should have a mesh sufficient to exclude Cytodex microcarriers (approx. 100 µm). An example of this type of system is described in reference 39. The culture can be contained in a chromatographic column (Pharmacia K 50/ 60) with medium circulated at 17 ml/min with a Pharmacia P-3 pump.

each cell type (section 4.3). Only those cell types which 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 23. After inoculation of cells into such a system it is necessary to have a static culture period. 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 (39) but is more difficult to use for general culture under aseptic conditions.

Perfusion culture
Cytodex 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 14. A suitable column for perfusion culture is a Pharmacia K 9/15 column (code no. 19-0870-01) fitted with an 80mm mesh nylon net (Pharmacia code no. 19-2268-01).

Dishes, tubes and multi-well trays
The surface area of cell culture Petri dishes and wells can be greatly increased by using microcarriers. The yield of cells from Petri dishes can be improved at least two-fold by adding microcarriers (5 mg Cytodex /ml) and the yield from wells can be increased five-fold using the same concentration of microcarriers. The microcarriers can be added as a suspension in the culture medium and the dish or well can be 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 so that extensive aggregation of microcarriers is avoided. Alternatively, the microcarriers can be added in PBS to the cell culture dish or well. After a few minutes the microcarriers attach to the culture surface, the PBS is removed and the dish or well can be 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 1.4.4). In this method, cells migrate from the confluent monolayer onto the microcarriers. The microcarriers can then be removed and used to inoculate other cultures or for biochemical studies.

Bacteriological Petri dishes can be used when the growth of cells is to be restricted only to the microcarriers. Such a culture is a useful first step when working for the first time with the microcarrier culture of a particular type of cell (section 3.1). For such a preliminary experiment the dish should contain 3-5 mg Cytodex/ml and is inoculated with approx. 1-2 x 10^5 cells/ml.

Culture tubes can also be used for microcarrier culture. However to ensure even growth of cells on the microcarriers the tubes must be kept in smooth motion. *Nilsson and Mosbach* (42) successfully used an end-over-end rotator for growing a variety of cells in tube microcarrier cultures.

3.2.3 Large scale microcarrier culture vessels

The requirements for large scale microcarrier culture equipment are similar to those for small scale cultures and 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 for large scale microcarrier culture (1, 4, 7, 32, 33, 40, 41, 47, 54, 55). However, to date the most suitable commercially available system has been a modified "Bilthoven Unit" (100) supplied by Contact Holland (Ridderkerk, Holland). Such units have been used for culture volumes up to several hundred liters (40, 41) for production of cell products such as interferon and virus vaccines.

Large scale equipment used for culturing microorganisms and suspension animal cell cultures is generally not suitable for microcarrier culture. Such equipment must be modified to account for the suspension properties of the microcarriers, the slow stirring speeds required and the culture procedures discussed in section 3.4. Optimal function of the large scale microcarrier culture process usually requires that specific design features are adopted for each application and situation. Further information on process design and equipment for large scale microcarrier culture can be obtained from Pharmacia Biotech.

3.2.4 Siliconizing culture vessels

Whenever glass surfaces are used with microcarriers 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.) which may be used for transferring or storing hydrated microcarriers. The best siliconizing fluids are those based on dimenthyldichlorosilane 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 which 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., Cat. no SL-2
Repelcote	Hopkins and Williams, Cat. no. 9962-70
Dimethyldichlorosilane	BDH, Cat. no 33164
Prosil-28	PCR Research Chemicals
Silicone Oil	Midland Silicones Ltd., Cat. no MS 1107, use as 2-5% (v/v) solution in ethyl acetate.
Siliclad	Clay-Adams, Cat. no 1950.

It is not necessary to siliconize polished stainless steel culture vessels.

3.3 Preparing Cytodex microcarriers for culture

The dry Cytodex microcarriers are added to a suitably siliconized glass bottle (section 3.2.) and are 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 (115°C, 15 min., 15 psi). It is not recommended that conditions for autoclaving exceed 120°C, 20 min., 20 psi. When hydrating Cytodex 3 initial surface tension may occasionally prevent wetting and sedimentation of the microcarriers. Should this occur, Tween 80 can be added to the PBS used for the first hydration rinse (2-3 drops, Tween 80/100 ml PBS).

Note. Cytodex 3 do not swell to the same extent as Cytodex 1 (table 4).

Prior to use the sterilized microcarriers are allowed to settle, the supernatant is decanted and the microcarriers are 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). Then the microcarriers are allowed to settle, the supernatant is removed and the microcarriers are 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.

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 they are allowed to settle, the supernatant is decanted and replaced 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 the dry microcarriers before swelling in sterile PBS with 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 makes dispensing the microcarriers into the fermenter a more simple step and reduces the risk of contamination.

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

3.4 Initiating a microcarrier culture

The initial phase of a microcarrier culture is usually the most critical stage in the culture cycle (38, 101). The success of the culture depends on correct procedures being followed when starting the culture and during the early phase of 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 conditions of inoculation and stirring speed.

When initiating a microcarrier culture the following points should always be considered. In most cases the conditions optimal for a given type of cell can be deduced from what is already known about its growth properties in culture (e.g. in Petri dishes or roller bottles) and also from preliminary experimens with microcarriers in Petri dishes (section 3.1.).

3.4.1 Equilibration before inoculation

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

The PBS in the sterile microcarriers should be removed by a rinse in warm culture medium (section 3.3). The culture temperature should be adjusted to a level optimal for cell attachment. In practice this temperature is usually the same as that use for the growth stage of culture (normally 35-37°C). Every attempt should be made to ensure that the culture pH is within the limits optimal for cell attachment (usually pH 7.1-7.4, section 4.4). Gas mixtures to be used during the initial stage of culture (section 4.3) 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 a longer time for equilibration. Small culture volumes (500 ml or less) can be equilibrated by incubating the culture vessel containing medium at 37°C

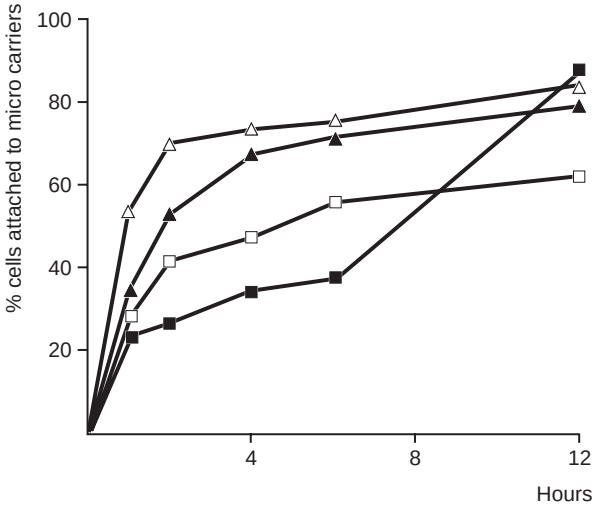


Fig. 24. The rate of attachment of various types of cells to Cytodex 1 microcarriers. All cultures were inocluated with 10^5 cells/ml and contained 5 mg Cytodex 1/ml. Stirring was continuous (50 rpm). —△— chicken fibroblasts, —▲— monkey cells (Vero), —❑— human fibroblast (MRC-5), —■— mouse fibroblasts (L-929). (From Pharmacia Fine Chemicals AB, Uppsala, Sweden).

and in an atmosphere of 95% : 5% Co_2 (section 4.3). After a few minutes the culture will be ready for inoculation. Stirring can be used to hasten the process of gas exchange. Equilibration of cultures with very large volumes may take 2-3 hours. The exact procedure used for equilibration should always be noted if reproducible results are to be obtained.

3.4.2 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 24 illustrates that under such conditions different types of cell 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 at the same time the cell-microcarrier mixture is contained in a reduced volume (e.g. in 1/3 of the final volume) then the cells have a greater chance of coming into contact with a microcarrier ant 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 cell types which have an intrinsically slow rate of attachment it is important that culture conditions allow the cells to attach as rapidly as possible.

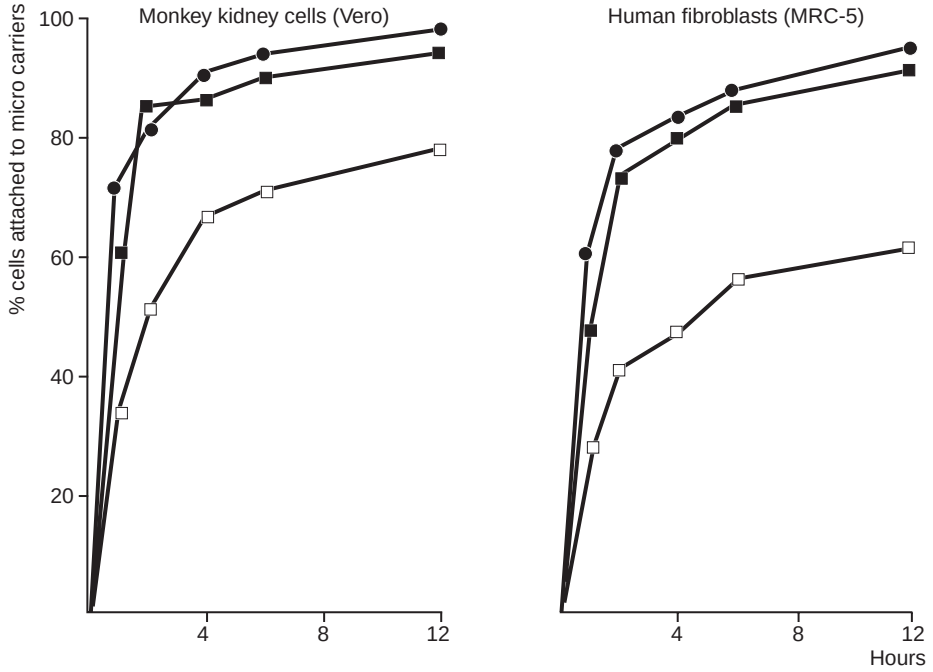


Fig. 25. The effect of initial culture procedure on the attachment efficiency of cells. Cultures containing 5 mg Cytodex 1/ml final volume were inoculated with 10^5 cells/ml final volume and were either stirred immediately (—❑—, 60 rpm) or cultured in a reduced culture volume with intermittent stirring(—■—). The modefied 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). Attachmment of cells to plastic Petri dished is indicated (—●—). (From Pharmacia Biotech AB, Uppsala, Sweden.)

Figure 25 shows the effect of using an attachment period with intermittent stirring and reduced initial culture volume on the rate of attachment on human fibroblasts and monkey kidney cells to microcarriers. For each type of cell both culture procedures use the same total number of cells and microcarriers but the modified procedure leads to a more efficient utilization on the inoculum. This procedure results in attachment efficiencies comparable to those observed in plastic Petri dishes.

The increased efficiency of attachment which results from initiating the culture in a reduced volume and with intermittent stirring results in an increase in cell yield at the plateau stage of culture (fig. 26). The improvements in cell yield when using this technique are most apparent where cultures are started with low inoculation densities.

This modified procedure at the initial stage of culture can be used for all types of cells and is particularly recommended when working with cells which have a low plating efficiency, (e.g. primary cell cultures and normal diploid cell strains) and if sufficient cells are not available to start the culture with optimum inoculation densities (section 3.4.4).

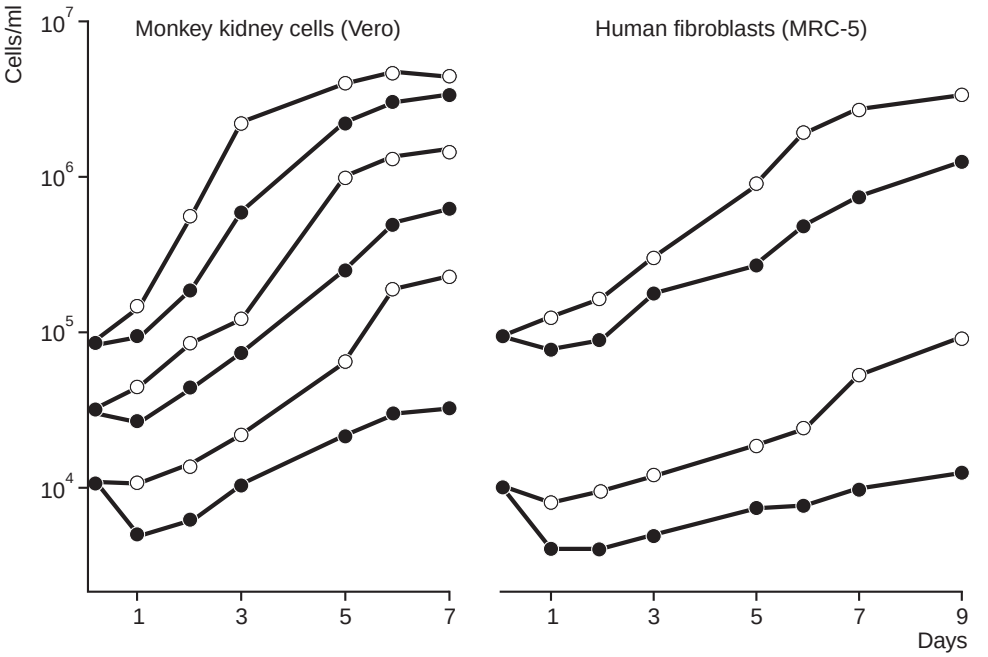


Fig. 26. The effect of inoculation density and initial culture procedure on the growth of monkey kidney cells and human fibroblasts on Cytodex 1 microcarriers. Cultures were inoculated with cells and stirred immediately in the final volume (—●—, 60 rpm) or were cultured in a reduced volume during the 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 (1981) 33*, by kind permission of the authors and publisher.)

The details of a modified initial culture procedure depend on the type of cell being cultured. A preliminary experiment with a stationary microcarrier culture in a bacteriological Petri dish will enable estimation of the time required for attachment of cells to the microcarriers and will also reveal any tendency for aggregation of cells and microcarriers under static culture conditions (section 3.1).

When starting most cultures the 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 3.4.1). After 3-6 hrs continuous stirring is commenced at a speed just sufficient to keep the microcarriers in suspension (section 3.5.1) 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 a low plating efficiency (less than 10%) or for cultures started with suboptimal numbers of cells (section 3.4.7) the culture volume can be maintained at 50% of the final volume for the first three days of culture and then fresh medium is added to reach 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 of culture is necessary for cell types which tend to clump when allowed to settle (e.g. primary chicken embryo fibroblasts). In such cases the initial stirring speed need only be approximately 25% of that normally used for the growth phase of the culture (section 3.5.1). *Griffiths et al.* (7) and *Moser et al.* (62) have observed that the modified initial culture procedure was essential for good results when growing human fibroblasts and heart muscle cells, respectively.

3.4.3 Concentration of microcarriers

The yield of cells from microcarrier culture is directly related to the surface area for growth and hence to 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 of microcarriers but these are cells which can grow at low culture densities. Provided an adequate supply of medium is ensured (section 3.5.2) and gas tensions and pH can be controlled (section 4.3., 4.4) it is possible to work with cultures containing more than 5 mg Cytodex/ml and in some situations it is possible to achieve 5-10 x 10^6 cells/ml. If consistent difficulties in maintaining culture conditions (pH and gas tensions) or in providing a sufficient supply of nutrients are encountered during the later stages of the culture cycle then decreasing the concentration of microcarriers should be considered.

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

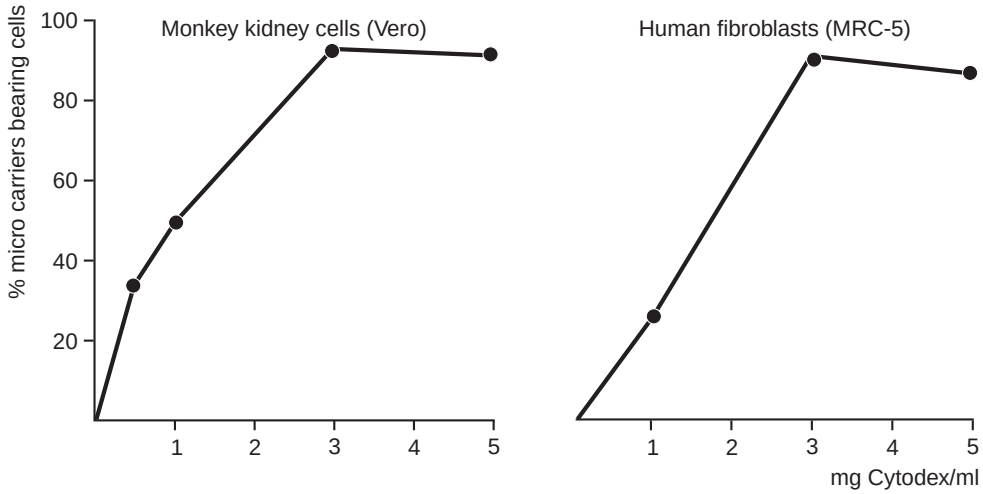


Fig. 27. The effect of microcarrier concentration on the proportion of microcarriers bearing cells at the plateau stage of culture. Cultures were inoculated with 5 viable Vero cells/microcarrier or 10 viable MRC-5 cells/microcarrier and stirred immediately at 60 rpm. The proportion of microcarriers bearing 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 wre not limiting cell growth. (From Pharmacia Biotech AB, Uppsala, Sweden.

concentration of microcarriers (fig. 27). 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 than the proportion of microcarriers bearing cells at the plateau stage of culture and the yield can be increased by using the modified initial culture procedure (section 3.4.2).

Provided a correct inoculation density is used (section 3.4.4) a concentration of 3 mg Cytodex/ml final volume is usually the optimal concentration for general microcarrier culture and results 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. In order to obtain the maximum yield of cells/cm^2 from cultures containing higher concentrations of microcarriers (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 3.5.2). This is simply because a given volume o medium can support the growth of only a finite number of cells.

As an approximate guide to expected cell yields it can be assumed that the culture has a density of 10^5 cells/cm^2 at confluence. This corresponds to 6 x 10^5 cells/mg Cytodex 1, 5.5 x 10^5 cells/mg Cytodex 2 and 4.6 x 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.

3.4.4 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 and 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 being 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 attachment of cells to Cytodex is similar to that observed in Petri dishes (fig. 25) the microcarrier cultures should be inoculation with approximately the same number of cells/cm^2 as used 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 (section 3.4.5) When inoculating a culture it is generally necessary to use more primary or normal diploid cells than established cells.

Inoculation density effects both the proportion of microcarriers bearing cells at the plateau stage of culture (fig. 28) and the yield from the culture (fig. 26). Figure 28 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* (14) reported that approximately

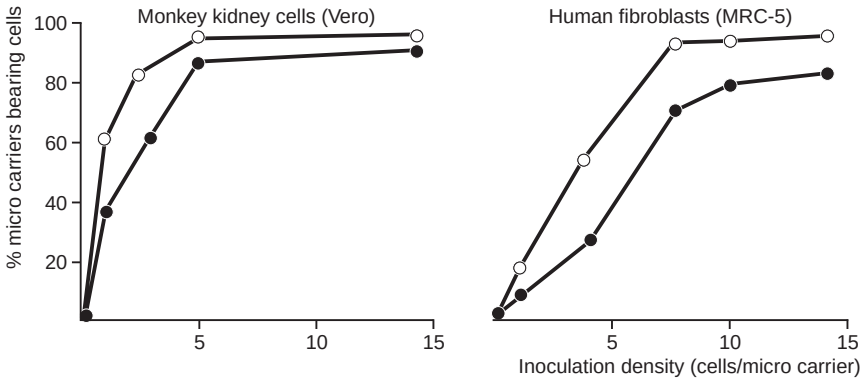


Fig. 28. The effect of inoculation density on the proportion of microcarriers bearing cells at the plateau stage of culture. Culture contained 3 mg Cytodex 1/ml final volume and were either stirred continuously at 60 rpm (—●—) or were started with a reduced volume and intermittent stirring before the culture was diluted to the final volume and stirred at 60 (—○—). Details of the modified culture procedure are given with fig. 26. The proportion of microcarriers bearing cells was determined after 7 days (Vero) or 9 days (MRC-5). (From Pharmacia Biotech AB, Uppsala, Sweden.)

5 cells/microcarrier were required when inoculating cultures with anterior calf pituitary cells. If sufficient cells are not available to inoculate the culture the modified initial culture procedure discussed in section 3.4.2 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. 28) and cell yields are increased (fig. 26).

Further details on inoculation density can be found in section 3.4.7.

3.4.5 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 which come 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 (101). When possible, microcarrier cultures should always be inoculated with cells taken from actively dividing cultures at approximately 70-80% confluence. In this way a greater percentage of cells in the inoculum can attach to the microcarriers and contribute to the growth of the culture.

The inoculum should be evenly dispersed and preferably a single cell suspension. Excessive centrifugation during concentration of the inoculum should be avoided since this leads to aggregation of cells and reduced viability after attempts at resuspension. Centrifugation for 5 min. at 200-300 g is normally sufficient.

Suspension of the inoculum in the medium to be used in the culture avoids dilution of medium components. Exposing the inoculum to sudden changes in temperature, pH or osmolarity should also be avoided.

3.4.6 Culture media during the initial culture phase

A general discussion on culture media can be found in section 4.1 and replenishment is considered in section 3.5.2 The nutritional requirements of cells are not necessarily the same throughout the culture cycle and for optimal result it may be desirable to alter the formulation of the culture medium at some stage in the culture cycle. This is usually necessary if the culture is to span a very wide range of densities e.g. from $5x10^4$ cells/ml at inoculation to $3x10^6$ cells/ml at confluence. In this section alterations to the medium during the initial stage of culture will be considered.

The main difference between culturing cells as monolayers on microcarriers or 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 . Nutritional requirements of cells growing under conditions of low density are usually more stringent than for cells growing under high densities (44) and the plating efficiency of cells can be improved by ensuring that the culture medium contains certain components in sufficient quantities. The necessity for supplementing the culture medium during the initial culture period depends on the culture medium, the inoculation density and the type of cell. For cells which 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 of common culture media 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 illustrates that the growth of cells in cultures inoculated with small numbers of cells is better when a more "complete" medium with a large number of components (Medium 199) is used rather than when a more sparse medium (DME, BME) is used. At high inoculation densities the plating efficiency of cells is improved and culture growth is greater in the medium with the highest concentration of amino acids and vitamins (DME).

Table 7. The effect of various culture media on the initial growth of monkey kidney cells (Vero) in microcarrier cultures. All media contained 10% (v/v) foetal calf serum and 5 mg Cytodex 1/ml. Cultures were stirred at 60 rpm from the moment of inoculation.

Inoculation density	Cells attached to microcarriers (%) of inoculum)					
	DME		BME		Medium 199	
	24 h	48 h	24 h	48 h	24 h	48 h
High $1.5x10^5$ cells/ml	210	460	180	350	150	280
Low $1.1x10^4$ cells/ml	60	70	45	55	95	145

Examples of media which are useful when culturing cells at low densities or cells having a low plating efficiency are Medium 199, McCoy's 5A, Ham's F10 or Ham's F12. The plating efficiency of cells inoculated at low density into Ham's F10 or

Ham's F12 media can be increased by doubling the concentration of amino acids and vitamins. More recent cloning media are described by *Ham and McKeehan* (44).

The difficulty with using cloning media is that they often lack sufficient reserves of nutrients to support growth of cells at high culture densities. This is particularly important if maximum yields are to be obtained at the plateau stage of culture when more than 10^6 cells/ml are present. A medium such as Medium 199 is relatively poor at supporting the growth of high densities of cells (table 7, fig. 34).

An alternative approach is to use a culture medium which can support the growth of high densities of cells and to supplement this medium to improve growth of cells at low culture densities. In general, components which are beneficial during the initial stage of microcarrier culture and which are not found in several common media include pyruvate, non-essential amino acids (182), adenine, hypoxanthine and thymidine. Table 12 present a useful general purpose medium based on DME which is supplemented for the initial stage of culture (59). The advantage of such a medium is that it has a higher concentration of amino acids and vitamins than most other media and can therefore support the growth of greater densities of cells. In addition the medium can be used for a wide variety of cells and when necessary, stock solutions of the above components can be added for the initial stage of culture.

Griffiths et al.(38) described a medium (MEM/MC) which was superior to others in its ability to promote attachment of human fibroblasts (MRC-5) to Cytodex 1 in large scale cultures. The modified medium was MEM with added glucose (1 mg/ml), glutamine (0.3 mg/ml), pyruvate (0.1 mg/ml), Eagle's non-essential amino acids (182), adenine (10 µg/ml), thymidine (10 µg/ml), hypoxanthine (3 µg/ml), inositol (2 µg/ml), choline chloride (2 µg/ml), tryptose phosphate broth (30 ml/liter, or approximately 1 mg/ml) and HEPES (5 mM). This medium was then supplemented with 5% (v/v) calf and 5% (v/v) foetal calf serum. MEM/MC resulted in attachment of cells to microcarriers nearly 2-fold greater than in CMRL 1066 and 4-fold greater than in standard MEM supplemented with serum. A shorter lag period was observed and after 24 h cell yield in MEM/MC was 5-fold greater than in MEM and nearly 2-fold greater than in CMRL 1066.

Increasing the concentration of the serum supplement can also improve the attachment and growth of cells during the initial stages of culture (section 4.2.2). Different types of serum supplement may also be used to promote cell attachment (section 4.2.2).

In cases of cells which are very difficult to grow in culture, or when cultures must be started at very low densities, conditioned medium may be used. The medium can be removed from actively growing or confluent cultures of, for example, fibroblasts, thoroughly clarified by centrifugation and then mixed (often 50:50) with a medium suitable for growing cells at clonal densities.

3.4.7 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 microcarrier culture procedures which are near optimal for that type of cell. This information often means that extensive preliminary experiments can be avoided and only minor adjustments to the culture procedure may subsequently be required to achieve the best results from the microcarrier culture. Comparative studies of the conditions required to culture a wide variety of cells in microcarrier culture have revealed that plating efficiency is one of the most useful growth parameters to consider when developing a culture procedure (38,39,101).

Plating efficiencies vary considerably between different types of cells and are a measure of the cell's ability to survive a susbculture 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 as intrinsically low plating effiency or if only small numbers of cells are available for inoculation then it is important to use culture procedures and conditions which enhance the plating efficiency.

Table 8 illustrates the relationships between plating efficiency and the initial culture variables. These relationships have been observed for a wide variety of cell types (38, 39, 101). 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%. In some cases the culture procedure may need to be modified because of 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 thoughout 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 on microcarriers (section 3.1).

Table 8. 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 3.4.4)	high (>10)	intermediate (5-10)	low (<5)
Initial culture volume (Section 3.4.2)	small (20-30% of final volume)	intermediate (30-60% of final volume)	large (100% of final volume)
Initial stirring speed** (Section 3.4.2)	static/intermittent	continuous (approx 10 rpm)	(approx 40-60 rpm)
Additional medium supplements (section 3.4.6)	required	advantageous	not required

* See text.

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

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 (%) which 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 subculturing ratio. A cell type which is subcultured routinely with a low split ratio (1:2) will usually require the initial microcarrier culture procedures necessary for a cell with a low plating efficiency (table 8). Cells which can be subcultured with a high split ratio (e.g. 1:20) have a high plating efficiency and modified initial culture procedures are not usually necessary.

Table 9 presents suitable inoculation densities for the microcarrier culture of some common established cell lines. The differences in inoculation densities reflect differences in plating efficiency. The inoculation densities refer to cultures stirred continuously from the moment of inoculation and if fewer cells are available, intermittent stirring in a reduced initial culture volume should be considered (section 3.4.2)

Table 9. A guide to comparative inoculation densities for some common established cell lines.

Inoculation density	Cell line
$2x10^5$/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
$8\text{-}9x10^4$/ml	Chang liver, HeLa, MDCK, MDBK, HT 1080, LLC-MK_2, LLC-RK, J111, L-132, Vero, Neuro-2a, RK 13
$5\text{-}8x10^4$/ml	CHO, HaK, Detroit 6, Detroit 98, Girardi heart, HEp^2, 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 3.4.2).

If the plating efficiency or routine subculturing split ratio of a particular type of cell is not known when 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 the size of the inoculum can be modified for subsequent cultures.

3.5 Maintaining a microcarrier culture

Once a microcarrier culture has been initiated certain procedures and precautions are required in order to maintain proliferation of cells and to achieve the maximum yield from the culture. 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 microcarrier culture cycle the changes in the density of the cell population are usually greater than 10-fold. Such growth leads to conditioning of the medium and thereby encourages the growth of cells in the culture until a saturation density is reached and there is density-dependent inhibition of proliferation (many established and transformed cell lines do not show suck inhibition). At the same time, oxygen and medium components are utilized and toxic products of metabolism accumulate. When maintaining a microcarrier culture these changes must be taken into account. In addition it should be kept in mind when optimizing the culture procedure that conditions optimal for growth of cells under low density (e.g. gas tensions, pH etc.) are not necessarily optimal at later stages of culture (section 4.3, 4.4, 5).

Note: The most important aspect of maintaining a microcarrier culture and obtaining 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 or if aggregation of the microcarriers occurs. Once the stage at which these changes occur is known corrective measures should be taken before such changes occur. It is often difficult to return to optimal conditions and obtain good results once large deviations have occurred and in such cases irreparable damage to the culture usually ensues.

3.5.1 Stirring speed

Although other culture systems can be used (section 3.2.2) the most suitable method for maintaining microcarrier cultures is in stirred suspensions. The purpose of stirring the culture is a) to ensure that the entire surface of the microcarriers is available for cell growth, b) to create a homogeneous culture environment, c) to avoid aggregation of microcarriers by cell overgrowth and d) to facilitate exchange of gases between the culture headspace and the medium.

In principle the 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 3.4.2), the culture should be stirred continuously. The rate at which the culture is stirred influences greatly the growth an final yield of cells (fig. 29) and this effect is related to an integrated shear factor (fig. 30). Slower stirring speeds reduce shearing forces on cells attached to the microcarriers but if the stirring rate is too slow, growth is reduced (fig. 29).

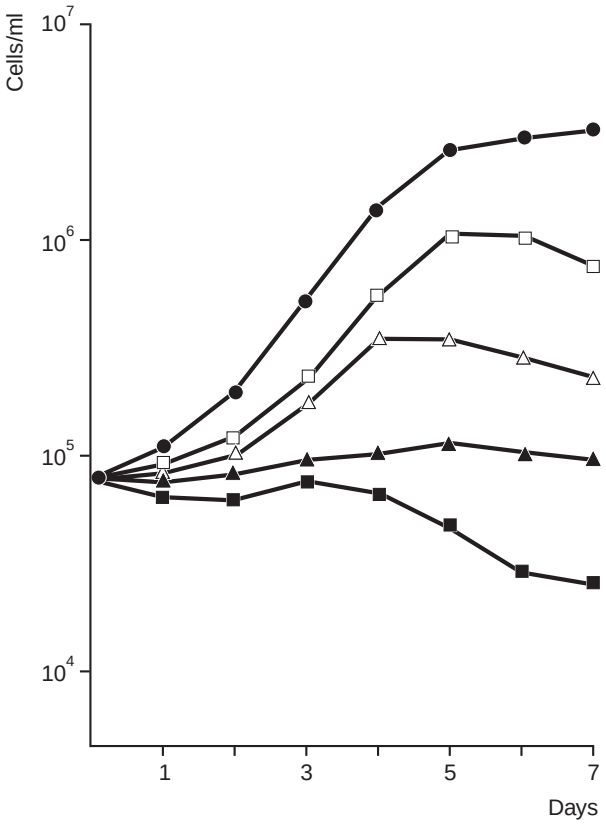


Fig. 29. Effects of stirring speed on the growth of Vero cells on Cytodex 1 microcarriers (30 rpm ▲, 40 rpm ❑, 60 rpm ●, 90 rpm△, 120 rpm ■). All cultures were 250 ml and contained 3 mg Cytodex 1/ml. Culture vessels were traditional magnetic spinner vessels and the cultures were stirred from the moment 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, 1980, pp 97, by kind permission of the authors and publisher.)

This effect is due mainly to inadequate gas diffusion and sedimentation and aggregation of microcarriers. If the stirring speed is too fast the less strongly attached cells (mainly mitotic cells) are dislodged 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 1.4.7). Excessive stirring speed cause a general loss of cells from the microcarriers and poor cell yields (fig. 29, 30).

Figure 30 illustrates that a population of cells growing on microcarriers is less sensitive to shear forces if initial attachment phase is included in the culture procedure (section 3.4.2). 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 the growth and plateau phase of the culture 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 such as many established or transformed cell lines. However, with respect to attachment the most critical stage of the cell cycle is during mitosis and most types of cells do not differ greatly with respect to strength of attachment during this stage. Therefore similar stirring speeds 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

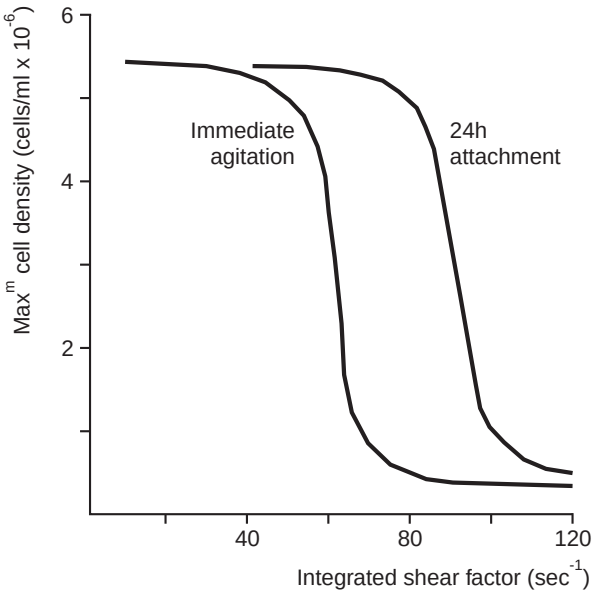


Fig. 30. The effect of shear force on the productivity of microcarrier cultures of chicken embryo fibroblasts. (Sinskey, A.J., Fleischaker, R.J., Tyo, M.A. et al. *Annals N.Y. Acad. Sci. 369 (1981) 47*, by kind permission of the authors and publisher.)

exchange and can be used to 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 of microcarriers during the later stages of the culture cycle (plate 5) and in these cases a slight increase in stirring speed will reduce the chance of aggregation. Increases in stirring speed must be considered carefully if there is any tendency for the cell monolayers to detach from the microcarriers. This detachment can be related to culture conditions and is discussed below.

The other aspect of selecting a correct stirring speed is the design of the stirrer and culture vessel and the volume of the culture. The optimal stirring speed when using traditional magnetic spinner vessels is usually 50-70 rpm (fig. 29) and speeds of 15-30 rpm are used with the modified spinner vessels or cultures stirred with bulb-shaped rods (section 3.2.2). Higher speeds are often required when using the same design of stirrer with culture volumes larger than 500 ml. A gradual increase in stirring speed to these levels over a few days is a desirable procedure. The stirring speed when working with large scale culture volumes also depends in the design of the stirrer. Progressive increases in stirring speed from 50 to 100-150 rpm during the culture cycle are frequently used for cultures of 100 liters or more (4, 10, *A. van Wezel,* pers. comm., 192).

With all types of stirring equipment it is important to avoid sedimentation of the microcarriers, particularly during later stages of the culture cycle. The accumulation of microcarriers which often occurs under the stirring axis in magnetic spinner

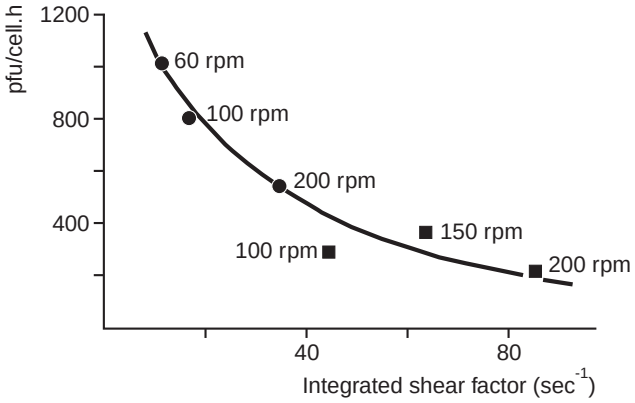


Fig. 31. The effect of shear force on the production of Sindbis virus from microcarrier cultures of chicken embryo fibroblasts. Virus productivity was averaged for the final 8 h of infection. Cultures were contained in magnnetic spinner vessels and stirring was with either a 4.4. cm (■) or 7.5 cm (●) impeller. (Sinskey, A.J., Fleischaker, R.J., Tyo, M.A. et al. *Annals N.Y. Acad. Sci. 369 (1981) 47*, by kind permission of the authors and publisher.)

vessels can be reduced by positioning the stirrer as close to the base as possible whilst 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 3.2.2, fig. 20)

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 a speed one half that used during the growth phase of the culture is optimal. Excessive stirring or shear forces result in decreased yields of viruses or cell products (fig 31).

3.5.2 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 which are depleted by cell growth.
b. Removing products of metabolism which inhibit growth or survival.
c. Assisting in control of pH.

Through careful planning of medium replenishment it is 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 tensions. Rapidly dividing cells and cultures at high densities require more frequent replenishment than low density cultures or slowly dividing cultures. Rapid cell division and high cell densities lead to depletion of medium components and a decrease in culture pH. At the same time meatabolites such as lactate, ammonia and even specific growth inhibitors accumulate (102, 183).

The ideal replenishment scheme is the one which results in the smallest fluctuation of nutrient concentrations and pH during the culture cycle. For this reason a continuous flow of medium is a preferred method for culture maintenance (103, 104). However, for small scale cultures or experiments with cell densities up to 3-5x10^6 cells/ml batch replenishment of medium is more convenient. The usual procedure is to start with replenishment of 50% of the medium volume every 3 days, If necessary, a modified scheme can then be developed in order to get the best yield form the culture. For example, it is common practice to observe the culture every day and to determine culture density (section 3.6). When samples are taken for these observations 10-20% of the medium volume can be replaced with fresh medium. In order to take advantage of conditioning effects the replenishment should not take place within the first two 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 to assist in the regulation of gas tensions and pH (sections 4.3, 4.4). 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 can be used (see below).

Note: Whenever fresh medium is added it should have the same temperature as the culture. The fresh medium should have a pH and osmolality optimal for cell growth (section 4.4, 4.5)

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 of culture, 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. (section 3.4.6, 6.4) can be omitted and greater economy can be achieved by reducing the concentration of the serum supplement (section 4.2.2). The type of culture medium can also be changed during the culture cycle. For example, if cultures are initiated with Medium 199, this medium can be replenished during the culture by addition of DME. This change is beneficial because although Medium 199 is good for growth at low culture densities (table 7), DME is superior for high culture densities (fig. 34).

Persistent difficulties with controlling pH at high cell densities can be overcome by modifying the carbon source in the medium, slightly increasing the oxygen tension or by using daily additions of glutamine in the presence of reduced concentrations of glucose (section 4.4.3).

If excessive aggregation of the culture occurs and cannot be controlled by adjusting the stirring speed (section 3.5.1), then the calcium and magnesium concentration in the medium can be reduced. A simple method for reducing the concentration of these ions is to use mixtures of media involving suspension culture versions of culture media (e.g. Spinner MEM). A 50:50 mixture of this medium with the usual culture medium normally overcomes difficulties with aggregation, without affecting cell growth.

When nutrient depletion is the factor limiting growth in a culture rather than accumulation of toxic metabolites or a decrease in pH, a different approach can be adopted.Growth limitation is caused by depletion of only certain medium components and table 10 lists the components in MEM depleted by the growth of human fibroblasts. Provided other factors are not limiting, replenishment of these components leads to continued cell growth. Figure 32 illustrates that a stock solution containing these components can be added to microcarrier cultures of chicken fibroblasts and cell yields can be as good as those achieved when complete medium is used for replenishment. This method provides for greater economy bur depends on good control of pH and definition of limiting nutrients.

Table 10. Depletion of nutrients from MEM by human diploid fibroblasts (MRC-5).

The data show the expected sequence of depletion in the presence of 10% (v/v) foetal calf serum and are taken from Lambert, K. and Pirt, S.J. *J. Cell. Sci. 17 (1975) 397-411.*

Nutrient	Concentration in MEM	Expected yield (cells/ml)
Glutamine	0.292 mg/ml	2.05×10^5
Cystine	0.094 mg/ml	2.15×10^5
Choline HCl	1 mg/ml	2.53×10^5
Glucose	2 mg/ml	4.04×10^5
Inositol	2 mg/ml	5.44×10^5
Pyridoxine	1 mg/ml	7.90×10^5

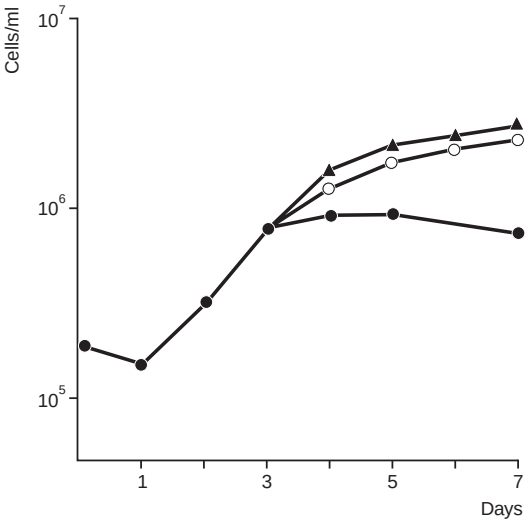


Fig. 32. The depletion of medium components during growth of secondary chicken fibroblasts in microcarrier culture. Cells were cultured in DME supplemented with 15 mM HEPES. 5% (v/v) calf serum, 1% (v/v) chicken serum and 1% (w/v) tryptose phosphate broth and containing Cytodex 1 microcarriers (5 mg/ml). 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 (1981) 33*, by kind permission of the authors and publisher.)

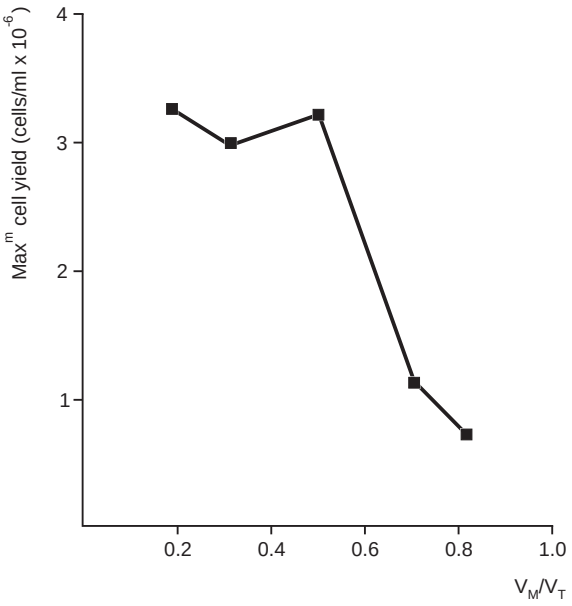


Fig. 33. The effect of culture volume and headspace volume on cell yields from a closed microcarrier culture system. Cultures were contained in traditional magnetic spinner vessels (Bellco) with a total internal volume (V_T) of 500 mls. Cultures of various volumes (V_M) were inoculated with Vero cells ($8x10^4$ cells/ml) and stirred at 50–60 rpm and 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 (1981)*, in press, by kind permission of the authors and publisher.)

All these aspects of medium replenishment are applicable to large scale microcarrier culture. An advantage with large scale cultures is that culture parameters such as pH and gas tensions are better controlled than with small scale closed culture systems and 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 in the ratio of the culture volume to the total internal volume of the culture vessel. Fig. 33 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 cell yield in closed culture vessels which 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 4.1, 4.3 and 4.4 respectively.

3.5.3 Maintaining cultures at confluence

Production from microcarrier cultures sometimes requires that monolayers of cells are maintained at confluence on the microcarriers for several days or even weeks. Fibroblast interferon, certain viruses and urokinase are examples of culture products where viable and functional monolayers of cells must be kept on the microcarriers for extended periods. *Mered et al* (3) describe the maintenance of monolayers of chicken embryo fibroblasts and Vero cells on microcarriers for periods of one 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 (48).

Similar procedures are used to maintain all types of cells at confluence on Cytodex and these procedures are the same as those used to maintain confluence in other monolayer culture systems. Cells which are contact-inhibited for proliferation (e.g. primary cells, diploid cell strains and several established cell lines) require stable culture conditions which promote viability and function of the quiescent population of cells. Such cells often form monolayers which become only weakly attached to culture surfaces when the 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, a reduced supply of serum supplements and by a consistent supply of fresh medium. For cell types which do not show contact-inhibition of proliferation. A reduction in the formation of multilayers of cells on the microcarriers and must also be accompanied by careful control of pH. Multilayers of cells growing on the microcarriers (plate 5) are very sensitive to changes in culture conditions and may detach after only small fluctuations in pH or nutrient supply.

The following are general points which should always be considered when maintaining microcarrier cultures at confluence.

- **pH.** It is most important to maintain optimum culture conditions, especially with respect to pH. Once a drift in pH occurs (usually a decrease) cells will tend to detach even after the pH has been returned to the optimal level. The most common cause for a decrease in pH at the later stage of the culture cycle is an accumulation of lactate (section 4.4.3).
- **Osmolarity.** When pH is being controlled by addition of acid/base or buffers it is important to avoid changes in osmolarity (section 4.5).
- **Serum concentration.** The most usual way of maintaining cultures at confluence is to reduce the concentration of the serum supplement (section 4.2.2). A reduction from the usual 5-10% (v/v) supplement to 2-5% (v/v) is required for cells which are contact inhibited for proliferation. For cells which continue to divide after confluence has been achieved lower concentrations of serum should be considered (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 and daily replacement of 10-20% 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 of the culture cycle and the main function of the medium replenishment is to control pH. Temperature shocks should always be avoided when replenishing the medium and all solutions should be prewarmed 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 (120,194). Hence glutamine-free medium and daily addition of approximately 0.1-0.2 mM glutamine assists in maintenance of 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 4.6.3).

- **Antibiotics.** When possible the concentration of antibiotics in the culture medium should be decreased for long-term maintenance of confluent cultures (section 4.7).

Details on the control of stirring speed and gas tensions can be found in sections 3.5.1 and 4.4 respectively.

3.6 Monitoring the growth of cells and microscopy

3.6.1 Direct observation by microscopy

Examining cells by microscope 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 and this can be accomplished by placing small pieces of broken coverslip between the slide and the coverslip. The formation of haloes occasionally occurs with phase optics and 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 3.6.4) and can then be stained (section 3.6.5).

Quantitation of 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.

3.6.2 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 are briefly washed in 2 ml of 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 a1:1 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 (5-10%, v/v) and the supernatant is pooled with the first supernatant. The cell suspension is centrifuged (300 g_{av}, 5 min., 4°C), the supernatant is discarded and the pellet is 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 haemocytometer or electronic counter and the concentration of the cells in the culture can be expressed per ml or per cm^{2} of microcarrier surface area (see table 4). Including trypan blue in the re-suspending solution allows estimates of cell viability to be made at the same time (section 3.7.9).

A similar method can be used when using collagenase in combination with Cytodex 3 microcarriers (section 3.7.2).

3.6.3 Counting released nuclei

A simpler way of monitoring cell growth is to count released nuclei as described by *Sanford et al* (105). In this method, modified by *van Wezel* (32), 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 g_{av}, 5 min.) and the supernatant is 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. Evaporation of the contents of the tube must be avoided 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 haemocytometer. The microcarriers in the sample do not interfere with the counting. The samples can be stored for up to one 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 5).

3.6.4 Fixing cells

When fixation and staining are necessary, e.g. for preservation of samples, cytochemistry, electron microscopy etc., any of the usual cell culture fixation and staining procedures can be used with Cytodex microcarriers.

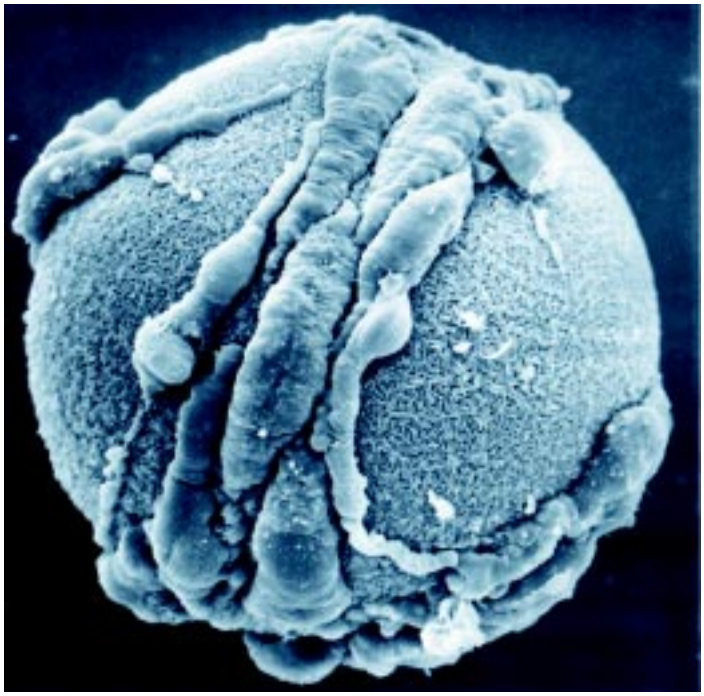
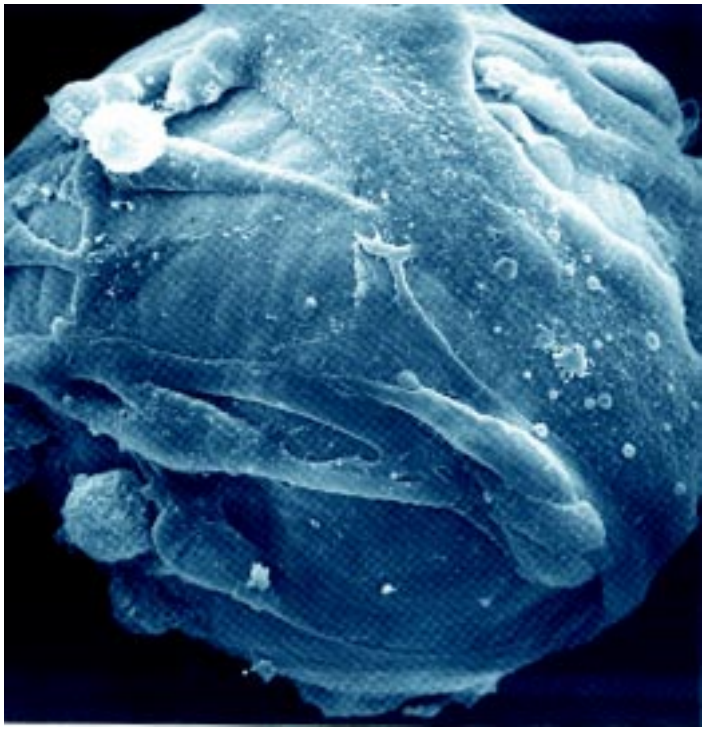


Plate 1: *Top:* Chicken embryo skeletal muscle cells (myoblasts) 2 days after inoculation onto Cytodex.



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, Scool of Medicine, Case Western Reserve University, Clevland, OH 44106, USA; work supported by the Muscular Dystrophy Assosiation and National Institutes of Health. Reproduced by kind permission.)

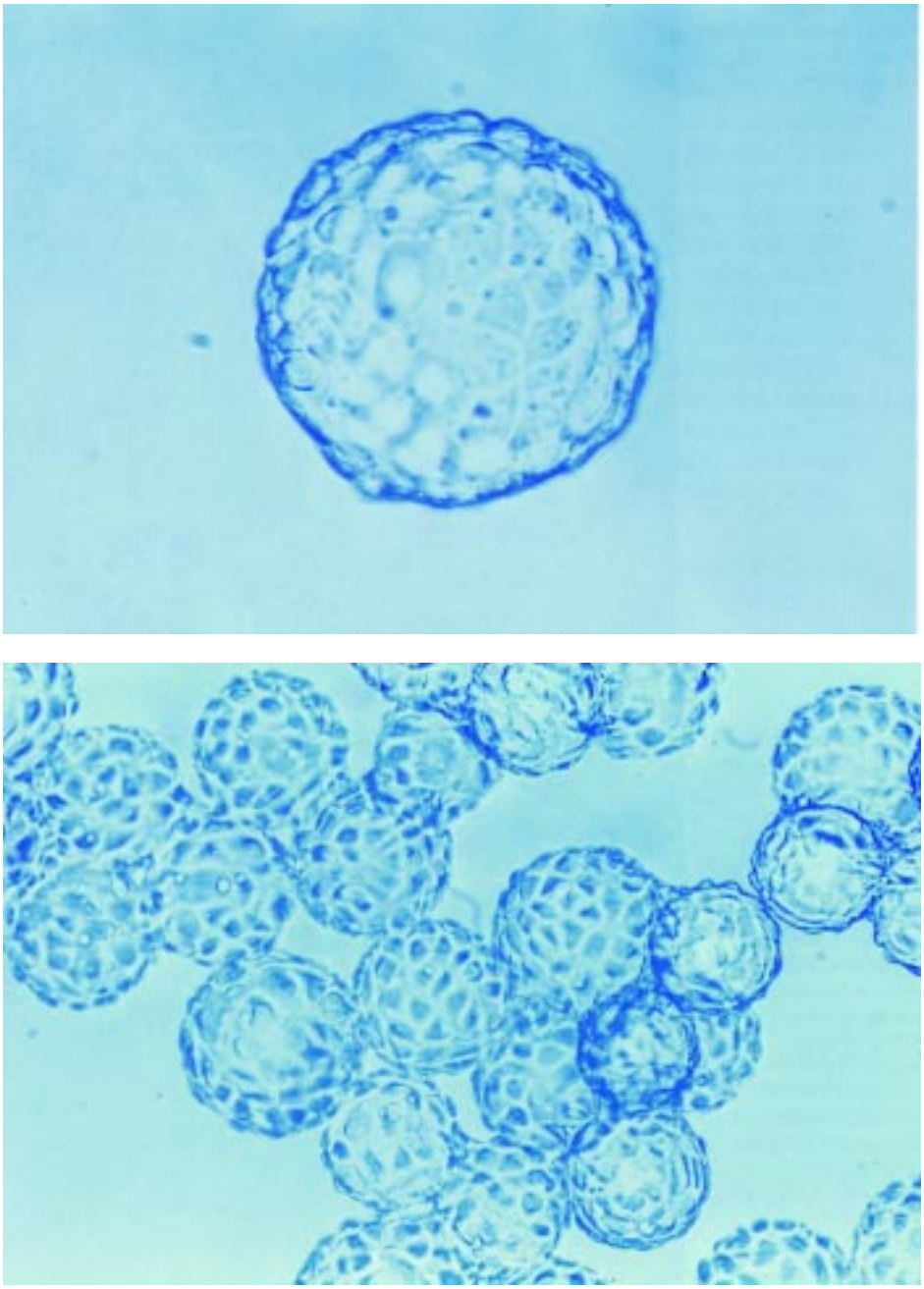
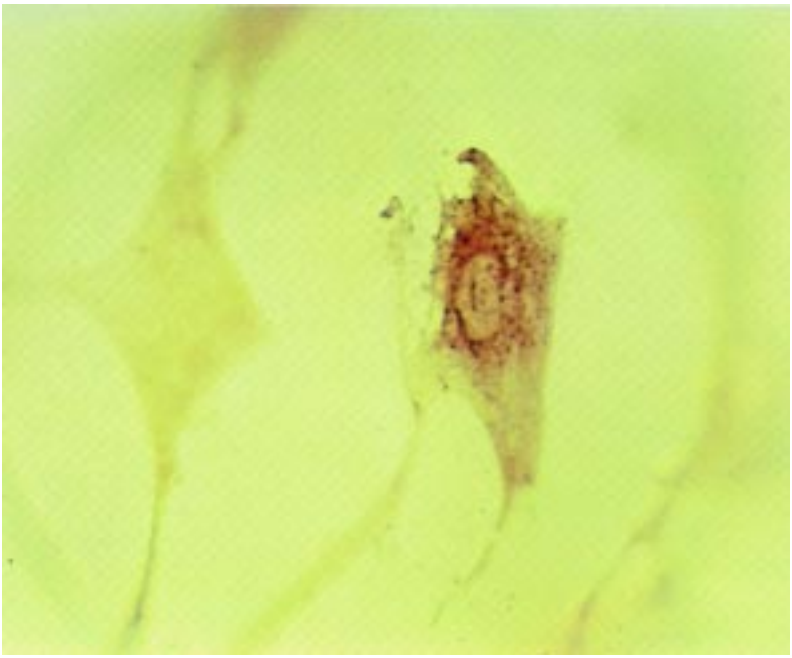


Plate 2: *Top:* A confluent monolayer of mouse brain capillary endothelial cells on Cytodex 3. *Bottom:* Bovine pulmonary artery endothelial cell growing on Cytodex 3. (Original photographs by Busch, C., Department of Pathology, University of Uppsala, Uppsala, Sweden. Reproduced by kind permission.)



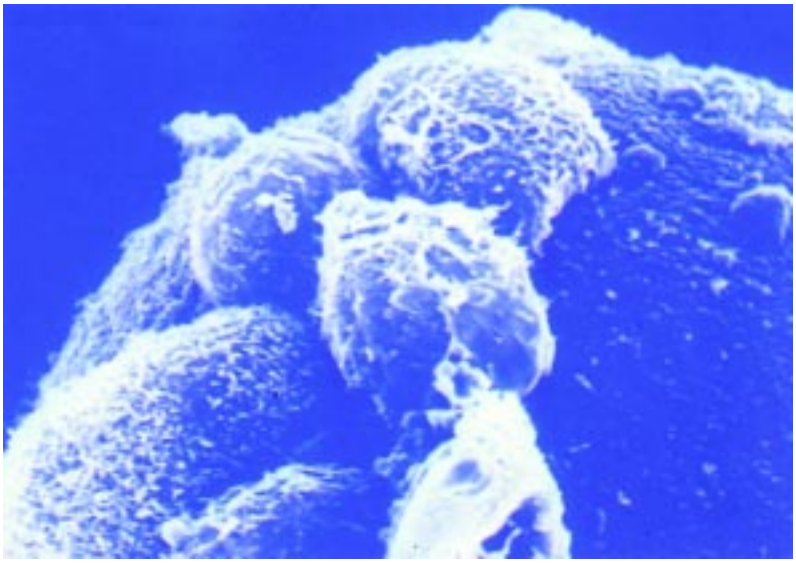


Plate 3: *Top:* Demonstration of insulin synthesis by immunostaining of foetal rat pancreas cell growing on Cytodex 3. (Original photographs by Bone, A., Swenne, I., Department of Medical Cell Biology, Biomedical Centre, Uppsala, Sweden. Reproduced by kind permission.)

Plate 4: *Bottom:* 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.)

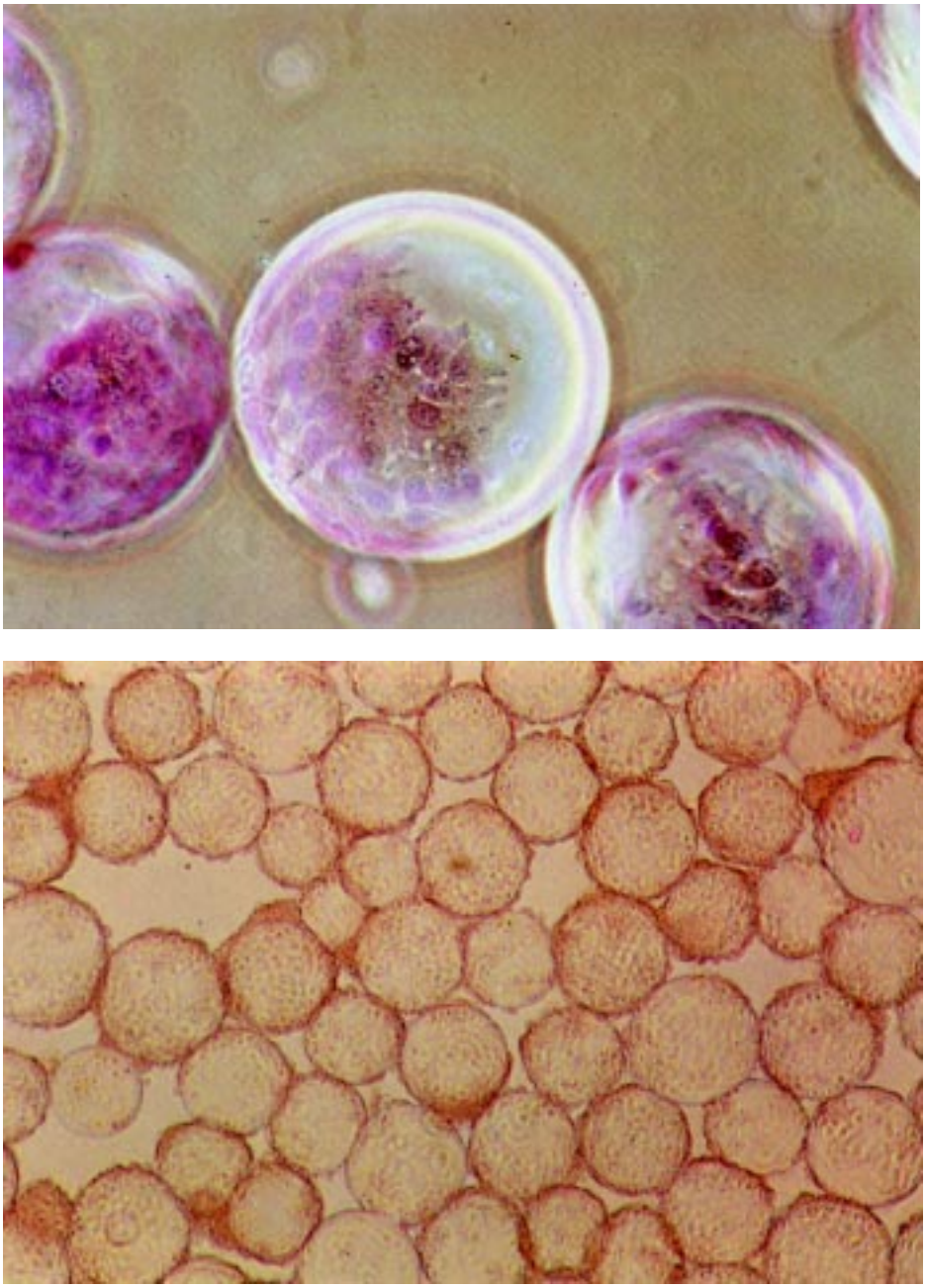


Plate 5: *Top:* Human cervical carcinoma cells (HeLa) growing on Cytodex microcarriers 3 days after inoculation. Note pronounced epithelial morphology.
Bottom: Confluent microcarrier culture of human glioma cells. The cells do not exhibit contact inhibition of proliferation and hence multilayers form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

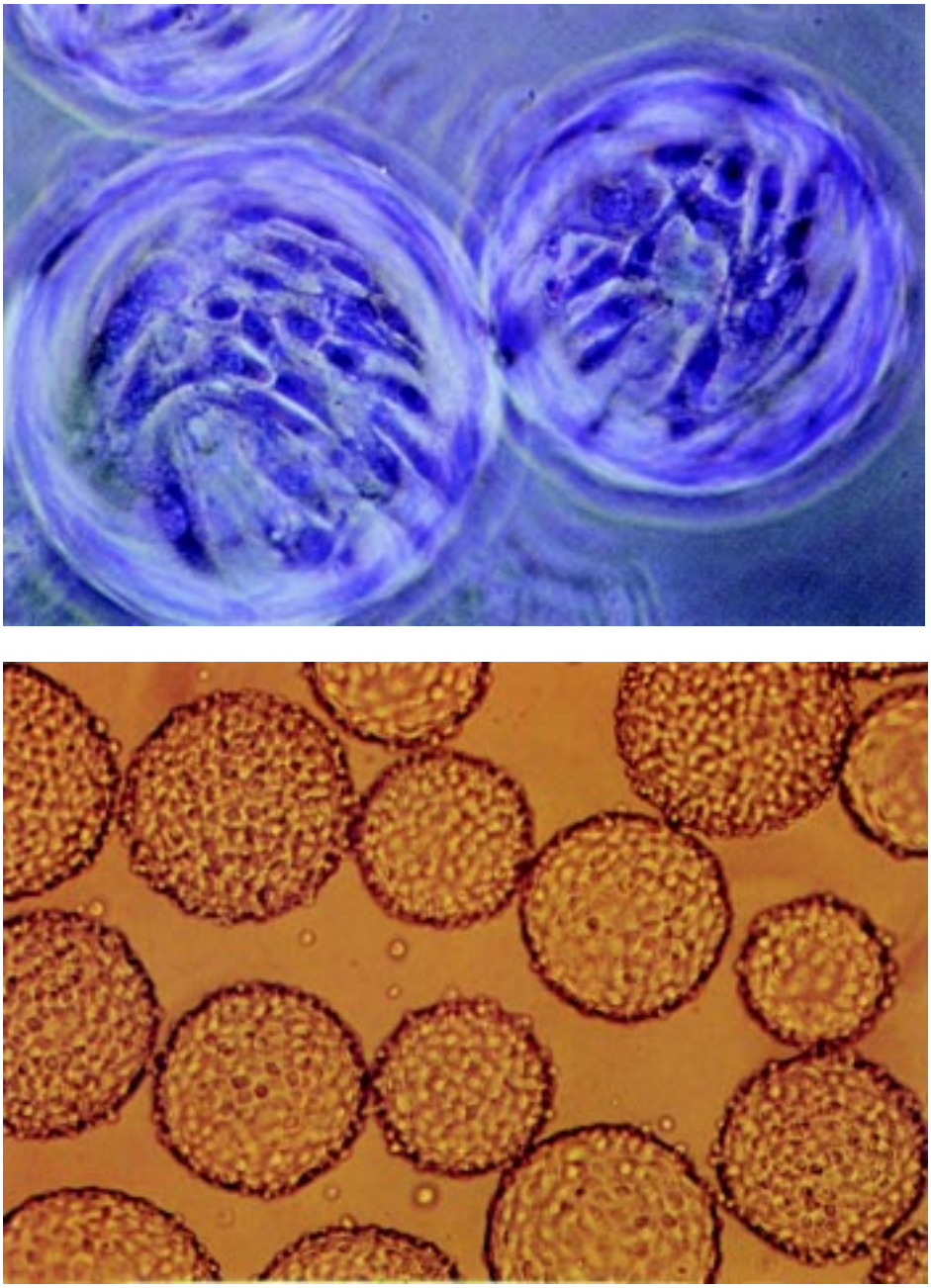


Plate 6: *Top:* Monolayers of diploid human embryo fibroblasts (MRC-5) on Cytodex microcarriers 7 days after inoculation.
Bottom: A confluent culture of human kidney cells (Flow 4000/Clone 2) on Cytodex 3.
(Original photographs by Pharmacia Fine Chemicals, Uppsala, Sweden.)

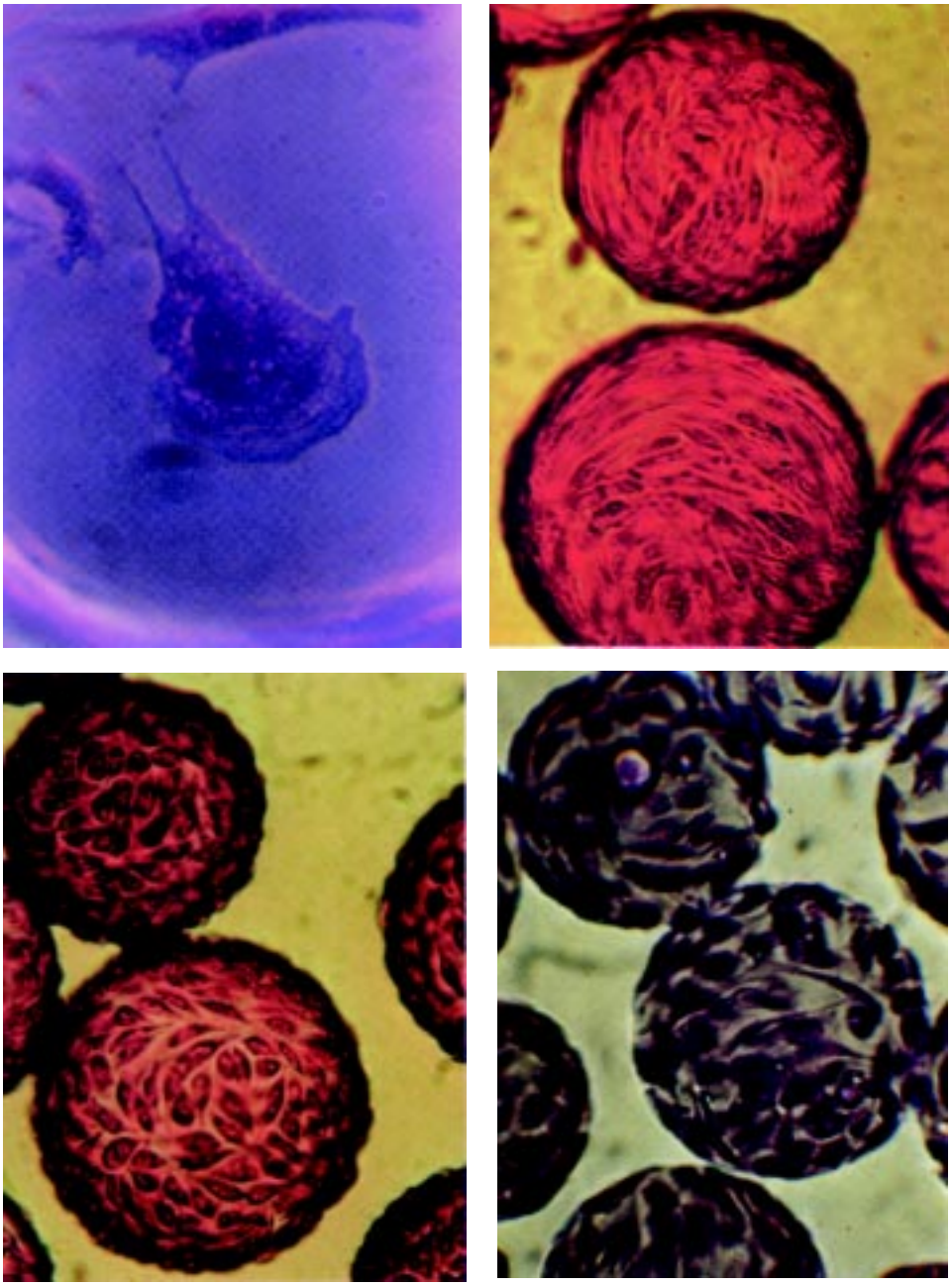


Plate 7: Examples of three common types of cells in microcarrier culture. *Top left:* Chicken embryo fibroblasts in motion 24 hours after inoculation. *Top righ:* Confluent monolayers of chicken embryo fibroblasts. Bottom left: Chinese hamster ovary cells (CHO) 4 days after inoculation, culture density $7x10^6$ cells/ml. Bottom righ: Diploid human foreskin fibroblasts (FS-4). These cells are often used for interferon production in microcarrier cultures (see refs 51, 58). (Original photographs by Tyo, M., Southern Biotech Inc., 3500 E. Fletcher Ave., Suite 321, Tampa, FL 33612, USA. Reproduced by kind permission.)

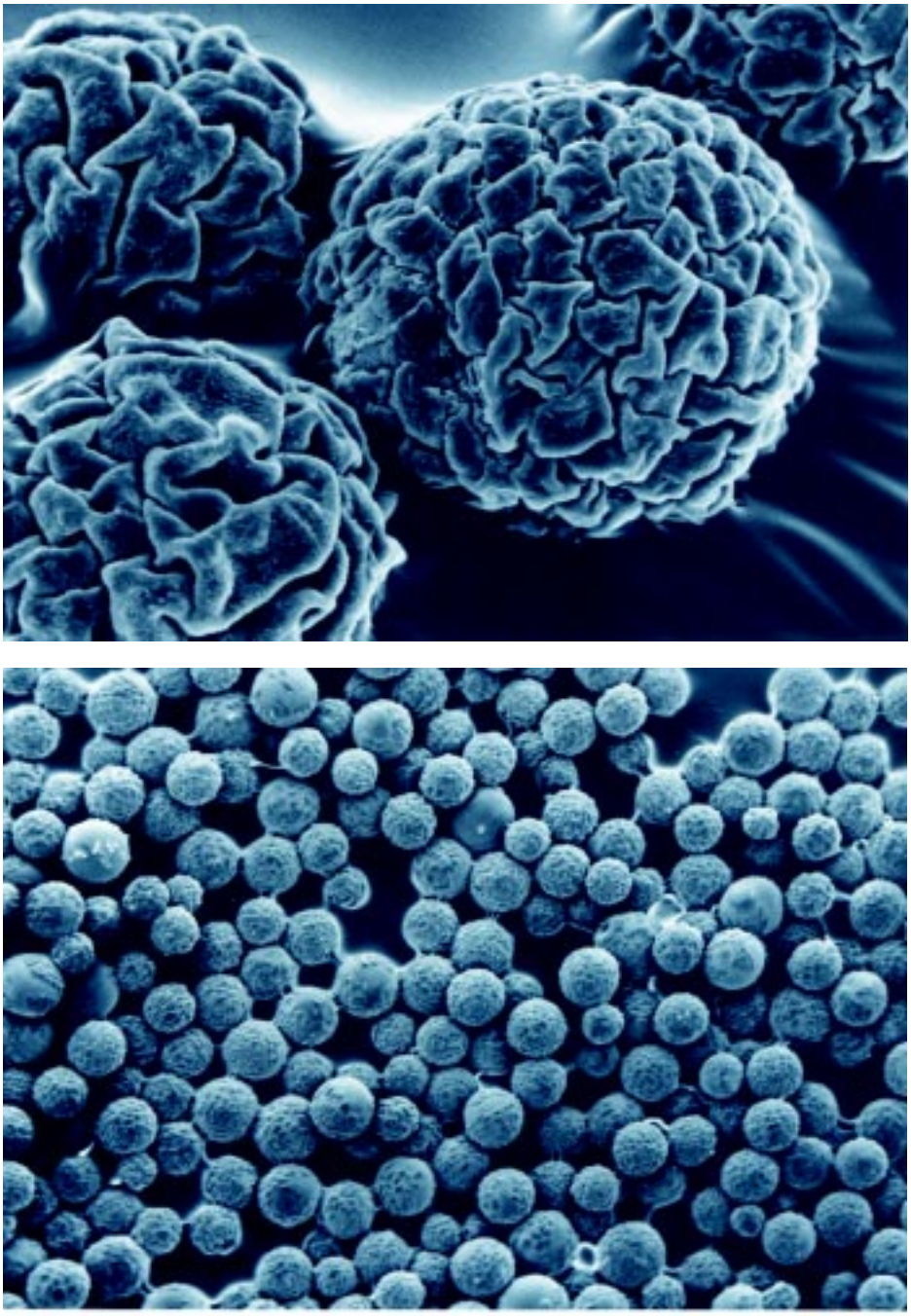


Plate 8: Scanning electron micrographs of pig kidney cells growing on Cytodex in cultures used for the production of foot-and-mouth disease vaccine. (Original photographs by Megnier, B., Tektoff, J., IFFA-Mérieux, 254 rue Marcel Mérieux, Lyon, F-69342 France. Reproduced by kind permission.)

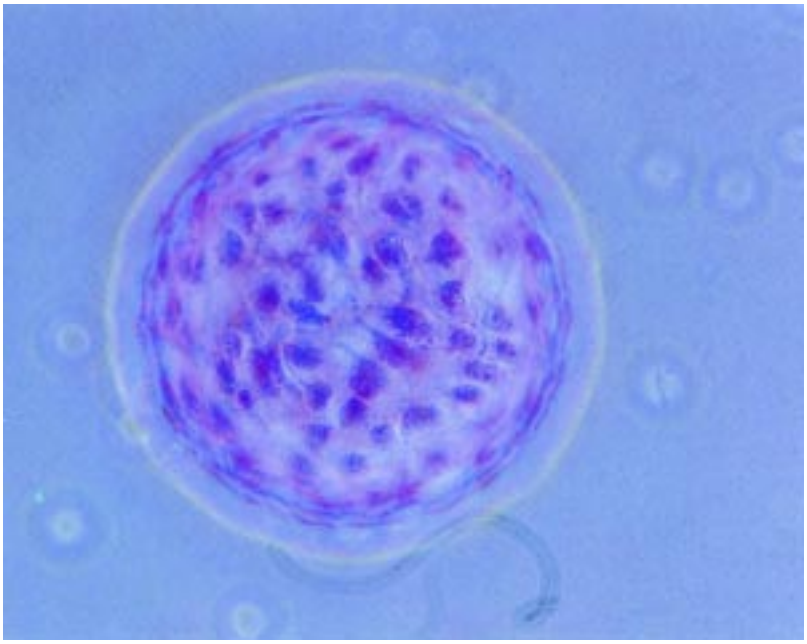


Plate 9: A predominantly epithelial monolayer of primary dog kidney cells growing on Cytodex. (Original photographs by Pharmacia Biotech Ab.)

The most common fixatives are methanol and ethanol. When using such alcohol's 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. the 50% alcohol is replaced by two 10 min. changes of cool 70% v/v) alcohol in PBS. Finally this fixative can be replaced by 70% (v/v) alcohol in water Alternatively, a modified Carnoy's fixative (3 parts methanol, 1 part glacial acetic acid and containing 2% (v/v) chloroform) can be used after the cells and microcarriers have been rinsed in PBS.

Better preservation of morphology will be achieved when aldehyde fixatives are used. Either 10% (v/v) formalehyde in PBS or 2-5% (v/v) glutaraldehyde in PBS can be used and the material should be fixed overnight at 4°C. Glutaraldehyde fixation results in the best preservation of morphology and the fixed material can be used for electron microscopy studies (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 (14). 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 propylene oxide which has been reported to alter the surface of microcarriers (55).When sections through cells attached to a solid surface are required, cells growing on Cytodex microcarriers are easier and more convenient to process than cells growing on the surface of Petri dishes or coverslips. When processing the microcarriers with cells attached for microscopy it must be remembered that the times taken for each step should allow for penetration of the microcarrier matrix by the solute or embedding agent- doubling the usual process times for embedding ensures good penetration of the matrix.

Examples of transmission and scanning electron microscopy of cells attached to Cytodex can be seen in figures 1 and 3 and plates 1, 4, 8. *Pawlowski et al* (61) used the following procedures for preparation of 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 $CaCl_2$/ 100ml) 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. The samples were dehydrated in a graded ethanol series and critical point dried with carbon dioxide. The coverslip were "sputter-coated" with gold for 1.5 mins at 25 mA and 1.5 kV (61; *R. Przybylski,* pers. comm., 193)

3.6.5 Staining cells

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

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 then followed by dehydratration 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.

Staining with Haematoxylin
The 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 haematoxylin 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. However, it should be noted 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 it is important 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 coveslips placed on the slide.

Plate 3 shows an example of immunostained cells attached to Cytodex microcarriers.

3.7 Harvesting cells and subculturing

Removal of cells from microcarriers is usually required when subculturing 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 then precipitation agent (usually 5-10% trichloroacetic acid) so that all un-incorporated isotope is washed from the microcarrier matrix.

Various methods can be used to remove cells from Cytodex and it is important to choose procedures which minimize damage to cells during harvesting. In most cases the usual 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 the microcarriers (section 3.7.2) 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.

3.7.1 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, the microcarriers are washed 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). The mixture should be stirred continuously in the culture vessel (fig. 20) at approximately 60 rpm for at least 10 min. The stirring speed may need to be increased or aspiration with a pipette may be required for some types of cell. When the cells have detached from the microcarriers the EDTA is neutralized by adding culture medium (100 ml/g Cytodex). The detached cells can be separated from the microcarriers as described in section 3.7.8.

In general, chelating agents alone are not sufficient for removal of most cell types and are therefore usually used in combination with protoelytic enzymes. Long periods of exposure to EDTA may be harmful to some fibroblast strains and this type of cell is rarely removed by EDTA alone.

3.7.2 Proteolytic enzymes

Enzymes are normally used for routine harvesting of a wide variety of cells from Cytodex microcarriers. In general trypsin, VMF Trypsin (Worthington), Pronaseâ (Sigma) or Dispaseâ (Boehringer) are used with Cytodex 1, Cytodex 2 and Cytodex 3 and in addition 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 which are sensitive to trypsin.

Trypsin

The stirring is stopped and the microcarriers are 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 3.7.8.

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). The 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 (156) 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 3.5.1).

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.

NaCl	122 mM	Phenol red	3.3 mM
KCI	3.0 mM	EDTA	0.02% (w/v)
Na_2HPO_4	1mM	Tris (hydroxymethyl) aminomethane	2% (w/v)
Glucose	4.0 mM	pH	7.8-8.0

To this solution is added trypsin at the usual concentration used for a given type of cell and 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 high content of DNA and RNA (106) 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 of trypsin. When possible, new batches of trypsin should be tested 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 (107-110). If subsequently studies require intact cell membranes or if rapid harvesting with maximum yields is required without impairment of cell viability then an alternative method of harvesting the cells must be used. The use of collagenase to harvest the cells growing on Cytodex 3 provides such a method and with this method the 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 (111) and can be used for the rapid harvesting of cells from collagen-coated surfaces. For example, *Michalopoulos and Pitot* (86) reported easy and rapid harvesting of hepatocytes from collagen-coated surfaces and *Sirica et al* (87) obtained 100% recovery of rat hepatocytes from a collagen surface within 10 minutes. In addition to simplifying harvesting of cells from cultures, a combination of collagenase and collagen-coated surfaces can be used for the selective removal of different cell types (78,112). 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.

The stirring is stopped and the microcarriers are allowed to settle. The medium is drained form the culture and the microcarriers are 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 g 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 3.7.8.

Collagenase requires Ca^{2+} and Mg^{2+} and therefore chelating agents should not be used during the harvesting step. When using the procedures described in section 3.7.8, steps directed at inactivating the collagenase are not usually required. the dilution factor and 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: The solution should be prepared in PBS or Krebs II buffer (47)and sterilized by filtration through a 0.2 mm sterile filter. Collagenase is usually used at a concentration of 100-500 mg/ml.

3.7.3 Hypotonic treatment

Incubation in hypotonic solution can be used for harvesting cells which 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. The Cytodex microcarriers with cells attached are washed twice in hypotonic saline (8 g NaCl, 0.4 g KCI, 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* (70) used hypotonic treatment to harvest CHO cells from Cytodex 1 microcarriers. Cell recoveries are usually less with this method than when enzymes are used. An advantage however of harvesting with hypotonic saline is that the harvesting does not involve exogenous protein.

3.7.4 Cold treatment

Incubation at low temperatures causes many types of cells to detach form culture surfaces. Cytodex microcarriers with cells attached can be incubated in culture medium without serum at 4°C for 8 h and a significant proportion of cells will detach from the microcarriers. 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. In general the use of temperature shifts for harvesting cells is associated with low viability and this method is best reserved for established cell lines when other methods are not desirable.

3.7.5 Sonication

Sonication alone cannot be used for harvesting intact cells from Cytodex microcarriers. In combination with the methods above low intensity sonication can be used to increase cell yields. Sonication can be used to rupture cells and leave membrane fragments attached to intact microcarriers (71;*S. Smit,* pers. comm., 191).

3.7.6 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 (113).

3.7.7 Modifications to harvesting procedures for large scale cultures

The above procedures have been described for small scale cultures. In principle exactly the same procedures 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. V*an Wezel et al* (40) 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* (114) describe the use of a narrow-bore tube for stripping cells from microcarriers. Using a 3.5 cm long capillary tube with a bore of 1.2 mm it was possible to obtain greater than 90% recovery of cells from the microcarriers (114).

Further information on procedures for harvesting cells in specific large scale culture situations can be obtained from Pharmacia Biotech.

3.7.8 Separating detached cells from microcarriers

There are several methods for separating detached cells from Cytodex.

- **Differential sedimentation.** Recovery of cells by differential sedimentation takes advantage of the fact that cells and microcarriers sediment at different rates. For routine harvesting and subculturing when maximum recovery is not required differential sedimentation is the most simple way of obtaining a preparation of cells essentially free from microcarriers. After completion of the harvesting steps described above culture medium is added (50-100 ml/g Cytodex) and the microcarriers are allowed to settle. After approximately 5 min. the culture vessel is tilted to 45°C and the cells can be collected into the supernatant. Better recovery can be achieved if the microcarriers are washed one more time with medium and the supernatant collected. The pooled supernatants can be used directly to inoculate the next culture. Alternatively, the products of the harvesting steps can be transferred into a narrow container with a high head, e.g. 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 of microcarriers. If greater recoveries are required then filtration should be used.

- **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 mm which 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 filter which is convenient to use 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, suitable nylon net can be obtained from Zurich Bolting (Rüschlikon, Switzerland) or Small Parts inc. (Miami, FA, USA).

- **Density gradient centrifugation.** Provided there exists a difference in density between the cells and the microcarriers density gradient centrifugation can be used to obtain a preparation of cells free from microcarriers. *Manousos et al* (55) used discontinuous density gradient centrifugation in Ficoll/Hypaque (density 1.077 g/ml) to achieve an efficient separation with no contamination of the cells by microcarriers. Ficoll-Paqueâ from Pharmacia Biotech is supplied sterile ready for use and can be used for this separation.

3.7.9 Measurement of cell viability

Exclusion of dyes provides a convenient measure of cell viability (115). 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.

Trypan blue solution is prepared in PBS (4 mg/ml). Approx. 0.9 ml of diluted cell suspension is mixed with 0.1 ml trypan blue solution. After 5 min. at room temperature the viable (unstained) and non-viable (stained) cells are counted in a haemocytometer. This counting can be done in connection with determination of cell concentration. Staining tests should be performed at pH 7.3-7.6.

3.7.10 Subculturing techniques

Subculturing cells from one microcarrier culture to another usually involves the steps discussed in section 3.4, 3.5 and 3.7. Cells harvested from one microcarrier culture can be used directly to inoculate the next culture containing fresh microcarriers. Transfer of a few microcarriers in the inoculum from the previous culture has no effect on subsequent culture development. For one subculture cycle it is possible when scaling-up to harvest cells from the microcarriers with trypsin, inactive the trypsin with medium containing serum and then to add fresh microcarriers. In this way the culture contains old and new microcarriers and procedures outlined in section 3.4 are used with corresponding increases in culture volume to maintain a constant concentration of microcarriers. The yield from such cultures is less 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.

An alternative potential method for scaling up is to simply add fresh microcarriers when the culture approaches confluence. *Manaus et al* (55) demonstrated that addition of a further 1 mg of microcarriers/ml of culture could be used to lengthen the life of RD cell cultures and to improve production of oncornavirus.

The success of this method of scaling-up depends on the ability of cells to move from the confluent microcarriers and to inoculate the microcarriers. Culture conditions need to be adjusted such 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 signigncant inoculation of the new microcarriers can occur (*P. Talbot*, pers. comm., 188). A reduction in the calcium concentration of the culture medium can be used to facilitate the transfer of cells between microcarriers (6).

Horst et al (72) described the use of Cytodex 1 for subcultivation 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 subcultivation can be found in section 1.4.4.

3.7.11 Re-use of Cytodex

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 washing steps are required in order to remove the debris remaining after the harvesting steps. The use of such procedures for washing Cytodex 1 is not recommended since the extreme of 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 the microcarriers for a third culture step has not been feasible with all cell types tested. For some cell strains re-use of the microcarriers is impossible.

Cytodex 3 microcarriers cannot be re-used when the cells have been harvested by enzymatic methods.

4. General considerations

4.1 Culture media

4.1.1 Choice of culture medium

A wide variety of different media can be used for the culture of any given type of cell. If culture medium support the growth of a particular type of cell in other culture systems then it will usually support the growth of cells on Cytodex microcarriers. Therefore, when selecting a medium for microcarrier culture the most suitable starting point is to use the medium which has previously been reported to support the growth of the particular type of cell.

Once the basic procedures for growing a particular type of cell 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 more rich medium is often needed for the initial stages of a microcarrier culture and 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 and stirring eliminates the formation of "micro environments" which occurs in static monolayer cultures. Since the cells have very little conditioning effect on the medium at this stage of culture one way of improving growth is to use a medium which contains components important for growth at low density. These components are particularly important for cells with low plating efficiencies (section 3.4.6).

The culture medium must have a large reserve of essential nutrients in order to support the growth of cells towards the end of the culture cycle when more than 10^6 cell/ml are usually present (section 3.5.2). Figure 34 illustrates 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 the growth of cells 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 (44).

Table 11 present a list of media which can be considered for culturing cells of different species. Although this list is far from complete and can be influenced by the tissue of origin of the cells, it is usually the case that a medium based on DME is suitable for the microcarrier culture of most types of cells. Section 6.1 lists the types of cells cultured on Cytodex microcarriers and a major proportion were cultured using DME as the base medium. In order to improve the growth of cells during the early stages of the culture cycle the DME can be supplemented with components which improve plating efficiency and growth at low densities (section 3.4.6). A medium which is recommended for general microcarrier culture is shown in table 12. This medium is based on DME and should be used if other formulations are not known to be more appropriate.

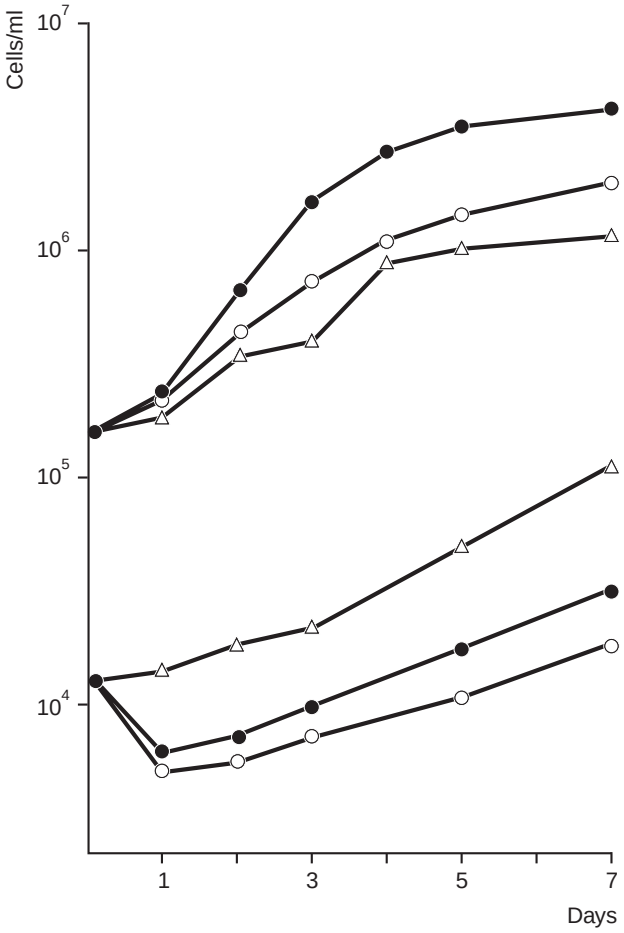


Fig. 34. The effect of various culture media on the growth of monkey kidney cells (Vero) on Cytodex microcarriers. (DME, ●; BME, ❍, Medium 199, △). All media were supplemented with 10% (v/v) foteal calf serum. Cultures contained 5 mg Cytodex 1/ml and were stirred at 60 rpm for the culture period. In all experiments 50% of the medium was changed on day 3. (Clark, J.M., Hirtenstein, M.D., *Annals N.Y. Acad. Sci. 369 (1981) 33*, by kind permission of the authors and publisher).

Table 11. Examples of culture media which can be used for cells of different species. The list is not exhaustive but covers the most commonly used media. The media are supplemented with serum and often with non-essential amino acids if not already present in the formulation. See *Ham and McKeehan* (44) for more information on formulations and section 6.1 for information on culture media used for microcarrier culture of specific types of cells.

General	Monkey Human	Rat Rabbit	Mouse	Chicken	Hamster
MEM	MEM	MEM	DME	DME	DME
DME	DME	5a	CMRL 1415	199	F10
199	199	F12	MCDB 202	F12K	F12
F12K	BME	F12K	MCDB401	MCDB 202	
MCDB 202	L15	MCDB 104		F10	
	5a			5a	
	RPMI 1640				
	CMRL 1969				
	MCDB 104				
	MCDB 202				
	IMEM-Z0				

Table 12. A general purpose medium for high yield microcarrier culture.

Inoculation medium (section 3.4.6)	
DME*	10^{-4}M
alanine	10^{-4}M
asparagine	10^{-4}M
aspartic acid	10^{-4}M
glutamic acid	10^{-4}M
proline	10^{-4}M
thymidine	$3x10^{-7}$M
adenine	10^{-5}M
hypoxanthine	$3x10^{-6}$M
pyruvate	10^{-3}M
HEPES	10^{-2}M
Serum	(see section 4.2)
Replenishment medium (section 3.5.2)	
DME	
HEPES	10^{-2}M
Serum	(usually lower concentration than in inoculation medium, see section 4.2).

A supplement of inositol 10^{-4}M and choline 10^{-4} is advantageous when maintaining high culture densities at low serum concentrations.

In certain cases the following components may be used to replenish the culture (fig. 32).

Cystine	30 mg/ml
Glutamine	0.3 mg/ml
Inositol	2 mg/ml
Glucose	2 mg/ml
Choline HCl	1 mg/ml

These additions are used only in the presence of low serum concentrations (5%, v/v, or less) and only when the pH is at a level suitable for cell survival and growth (section 4.4.1).

Maintenance medium
Based on replenishment medium, occasionally with modifications (see section 3.5.3, 4.1.2, 4.3.2).

* DME contains the non-essential amino acid serine.

When culturing cells with low or intermediate plating efficiency (i.e. less than approximately 30%) the basic DME medium is supplemented for the initial stages of culture until the medium is replenished. Addition of non-essential amino acids is the single most important supplement for improving plating efficiency and growth in cultures with low densities (fig. 35). Whenever cultures are initiated at densities of less than approximately $7x10^4$ cells/ml in cultures containing 3 mg Cytodex/ml, a medium such as that described in table 12 should be used for the initial stages of culture. Modifications to the initial culture procedure will also improve the yields from the culture (section 3.4.2).

Microcarrier cultures are usually replenished with the basic formulation of DME. Under conditions when very high cell densities are being cultured (more than approximately $3x10^6$ cells/ml) or when the cultures are rapidly dividing during the later stages of the culture cycle e.g. many established cell lines) the replenishment

medium is supplemented with additional inositol and choline. These increased concentrations of inositol and choline are important if the replenishment medium contains only low concentrations of serum (section 3.5.2). Increasing the concentration of these components can reduce the frequency or extent of medium replenishment, provided other factors do not become limiting (section 3.5.2 and 4.4).

The medium described in table 12 should be supplemented with the usual concentration of serum (section 4.2.2). Buffer systems for the control of pH are discussed in section 4.4.2.

4.1.2 General comments on components of culture media

While the requirements for growth of cells in microcarrier culture are similar to those for other monolayer methods it is important to consider several components of media if the culture conditions are to be optimized.

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 35. There is an extremely rapid utilization of amino acids during the lag phase of growth and a long lag phase will cause a reduction in the maximum cell population when amino acids are growth-limiting (180). 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 the plating (116, table 10). 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 (46). It is usually these amino acids which are depleted first by a variety of types of cells.

Deficiencies in supply of any one of the essential amino acids stresses cultured cells and may inhibit cell division, induce chromosome damage, and increase lysosomal activity and cell size (117-118). There is a long recovery period after such restriction (see 118). Restriction of amino acid supply is a frequent occurrence with many culture procedures and it is important to avoid limiting concentrations or imbalances in amino acid levels if high cell yields are to be achieved. One way of avoiding imbalances or wide fluctuations in the levels of amino acids (or other medium components) is to follow a strict scheme for replenishment of the medium (section 3.5.2).

It is important to note that additions such as lactalbumin hydrolysate often provide unphysiological mixtures of amino acids and may even result in changes in karyotype (181). These complex mixtures should be used with caution when working with many primary cells and cells with low plating efficiencies which have not been adapted to growth in medium containing these supplements.

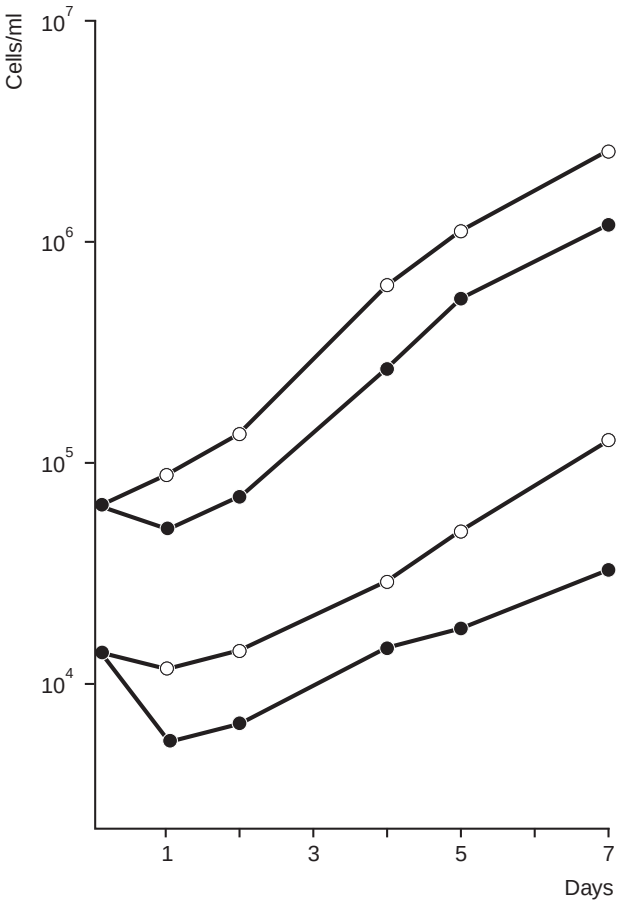


Fig. 35. The effect on nonessential amino acids on the growth of monkey kidney cells (Vero) on Cytodex microcarriers. The culture media were DME supplemented with 10% (v/v) foetal calf serum (●) or DME supplemented with 10% (v/v) foetal calf serum, alaine, aspargine, asparcit acid, glutamic acid and 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).

Since large imbalances or excessive concentrations reduce growth, amino acids should not be supplied at levels which 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 those in the original formulation. Glutamine plays a vital role in metabolism and is a precursor for nucleic acid synthesis and also an important carbon source. Glutamine is the most unstable of amino acids and decomposes in culture medium to form pyrrolidone carboxylic acid and ammonia (120). Regular addition of glutamine can be used to replenish the culture medium and compensate for the decomposition. Increasing the concentration of glutamine to 2.5 mM during the initial stages of culture usually results in better cell growth, especially at low cell densities (121). 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 (122).

The optimum concentration of cystine depends to a large extent on the serum concentration and batch (118) and if low concentrations of serum are used the cystine concentration may need to be reduced. For general microcarrier culture the cystine levels described in the original formulations of the various culture media are adequate but for optimization of a particular process it may be valuable to examine the effect of different concentrations of cystine. When replenishing the culture medium without complete replacement new cystine can be added approximately every 3-4 days at the concentration described in the formulation (section 3.5.2, figure 32).

Nucleic acid precursors
A supply of components such as adenosine, guanosine, cytidine, uridine (each 10^{-5}M) and thymidine ($3x10^{-7}$M) is often beneficial, particularly in cases where 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. Therefore in some cases, e.g. primary cells or when culturing normal diploid cells, it may be necessary when using Medium 199 to add extra folic acid or thymidine (123). A simplified supplement of nucleic acid precursors for a general purpose medium is included in the medium in table 12.

Carbon sources and lactate
The growth of cells in culture depends on a source of carbon. In most of the commonly used culture media this source of carbon 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 the concentration of glucose falls below approximately 20mM, detachment of cells occurs (124). Media containing glucose should be supplemented with pyruvate (1mM) for the growth of cells under conditions of low density (table 12).

The type of carbon source in culture medium influences the formation of lactate. The use of different carbon sources in controlling pH is discussed in section 4.4.3.

Vitamins and choline
Addition of retinoids can promote adhesion of cells which adhere weakly to substrates (125). Retinol or retinoic acid can be added at up to 1 mg/ml for the improvement of cell adhesion and their effect may be due to influences on synthesis of specific glycoconnjugates of the cell surface (125).

A sufficient supply of choline is vital to successful microcarrier culture. The main fate of choline is incorporation into membrane phospolipids and when choline levels decrease, the resulting pertubation of membrane composition causes rounding of cells and decreased adhesion (126). It is for this reason that the medium in table 12 has increased levels of choline for later stages of the culture cycle. Additional choline is important when using low concentrations of serum (less than 5%).

Culture media should not contain ascorbic acid when producing RNA tumour viruses (127). In contrast, ascorbic acid enhances the yield of interferon from microcarrier cultures (59).

Polymers
A high molecular weight component may be a necessary supplement for the culture of some types of cells at low cell densities (128). When working with cells possessing very low plating efficiencies (less than 1%) and low inoculation densities (less than $8x10^4$ cells/ml) it is beneficial during the early stages after inoculation of the culture to include a polymer in the medium. The polymers improve cell survival but have no effect on growth. Suitable polymers are Ficollâ 400, Dextran T-70, Dextran T-500 (all available from Pharmacia Biotech) or methylcellulose and 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 replacement of medium is not possible, carboxymethyl cellulose (0.1 mg/ml) can be added to reduce the effects of these toxic components. Ficollâ 400 can also be used to reduce turbulence when culturing cell types which attach very weakly to culture surfaces, e.g. some established cell lines such as lymphoblastoid cells.

4.1.3 Practical aspects of culture media
The water and reagents used for preparing culture media should be of the highest possible purity. All glass bottles used for storage of medium should be of high quality glass with a low content of heavy metals and should be well washed (129). In order to approve reproducibility of microcarrier cultures 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 the many suppliers of culture media.

4.2 Serum supplements

4.2.1 The purpose of serum in culture media
A serum supplement in usually an essential component of culture media for animal cell culture and in the absence of serum most cells fail to proliferate. Sera used to supplement culture media come form a variety of sources and are used at concentrations ranging from 0.5% to 30% (v/v). In microcarrier culture it is usual to use a serum supplement of 5-10% (v/v) for general purpose cultures. While culture media are chemically defined the serum supplement is undefined, especially with respect to those components responsible for promoting growth of cell cultures. The serum serves two vital functions. Firstly in assists attachment of cells to the culture surface, probably by supplying exogenous glycoproteins involved in the attachment process. Secondly, growth factors and hormones in the serum promote proliferation of cells. The serum also has a protective effect on cells in culture and enhances viability. A further function of the serum is to provide protease inhibitors which inactivate the trypsin used in routine subculturing procedures (section 3.7.2).

4.2.2 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 in culture.

Foetal calf serum has been a common supplement because of a high fetuin content and a low content of gamma-globulin and fat (130). This serum often has the ability to promote the growth of more fastidious types of cells. Foetal calf serum is also unique in having high levels of biotin (131) and therefore provides a source of this growth promoting component which is not present in some media formulations (e.g. MEM, DME, L-15).

The main disadvantage of foetal calf serum is that it is much more expensive than many other sera and supply is often limited. Alternative sera are required for microcarrier cultures of several hundred liters where foetal calf serum could be at a concentration of 5-10% (v/v). A further disadvantage of foetal calf serum is that it is one of the most variable sera with respect to hormone levels (132, table 13), and also contains significant levels of arginase, an enzyme which can deplete the medium of the essential amino acid arginine (130).

Figure 36 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 36 may not be the same for all types of cells it illustratese the principle that for the culture of any particular type of cell a variety of different sera should be tested.

Table 13. Cell growth and variation of components in batches of foetal calf serum from commercial suppliers. The data are complied from several sources (132, 138-141). A measure of variations is provided by CoV (standard deviation/mean x 100).

	Unit	Range	CoV (%)
Plating efficiency*	%	0.6-19.6	52
Cell growth**	10^{-4} cells/cm^2	0.57-19.5	66
Protein	g %	1.68-5.30	10
Haemoglobin	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/liter	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 foetal lung cells after 72 h.

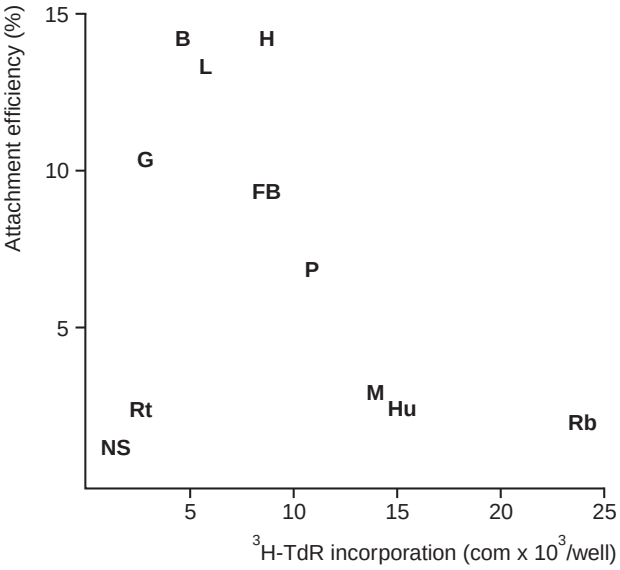


Fig. 36. The effect of various sera on the attachment efficiency and thymidine incorporation in cultures of mouse mammary epithelial cells. Cells were cultured in medium supplemented with 20% serum. Abbreviations;
B-bovine, L-lamb, H-horse, G-goat, FB-foetal bovine, P-pig, M-mouse, Rt-rat, Hu-human, Rb-rabbit. (Feldman, M., Wong, D., *In Vitro 13 (1977) 275*, by kind permission of the authors and the publisher.)

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 low concentration (5-10%) favor proliferation and differentiation (133). Certain sera may inhibit differentiation of cells in culture. The inhibition of chondrogenesis in chicken limb-bud cells by mouse serum (134) illustrates that some sera have very specific effects. *Harrington and Godman* (135) describe a factor in the alphaglobulin fraction of some sera which 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 purposes requires a flexible approach towards selection of serum supplements. Several different strategies can be adopted and by using one or more of these it is possible to reduce considerably the cost of serum supplements whilst still maintaining high cell yields

Reducing the serum concentration

The concentration of the serum supplement can often be reduced below the levels traditionally used for a given type of cell. Maintaining optimal culture conditions (e.g. pH and gas tensions) is particularly important if high cell yields are to be obtained in the presence of lower serum concentrations. *Giard and Fleischaker* (58) reported that 5% (v/v) foetal 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 concentrations.

Unless special media are used (44), 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 of the culture. 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 serum supplement depends on the stage of the culture cycle and is related to the functions of the serum.

The concentration of the serum need not be constant throughout the culture cycle. A typical procedure in microcarrier culture is to use a 10% supplement for the first three days of culture (or until the culture contains approximately 1-3 x 10^5 cells/ml) and then to use a medium with only 5% serum for replenishment (section 3.5.2). Once the culture has reached confluence the concentration of serum is reduced further (section 3.5.3), often as a low as 0.5%. *Horng and McLimans* (13, 14) noted that shedding of confluent monolayers could be avoided by decreasing the 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 replace to a large extent the growth promoting properties of serum, particularly when growing established cell lines and some primary cells. These undefined mixtures of amino acids and polypeptides are often used at a concentration of 0.25-0.5% (w/v).

Changing to another type of serum
In many cases newborn or donor calf serum can replace the more expensive, less plentiful foetal calf serum supplement. Only a few types of cells require foetal calf serum and include amniotic cells, biopsy material and other primary cultures where the density of cells is very low. Other types of cells or cultures may show better growth in medium supplemented with foetal 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.

Blending different sera
By blending different sera it is possible to reduce the cost of the serum supplement and still maintain high yields form microcarrier cultures. A mixture of foetal calf serum and newborn calf serum (50:50) will often result in cell yields identical to those obtained in the same concentration of foetal calf serum (fig. 37). Various sera differ in their ability to assist attachment and promote cell division (fig. 36). Therefore mixing of sera known to support these individual functions can result in improved growth of the culture (136).

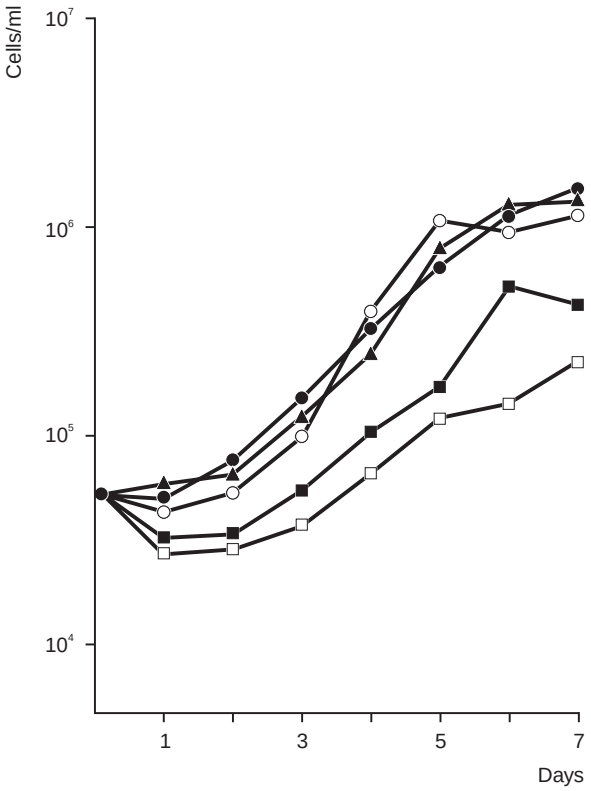


Fig. 37. The effect of various types of serum supplement on the growth of monkey kidney cells (Vero) in microcarrier cultures. Cells were cultured in modefied DME medium (table 12) containing 3 mg Cytodex 1/ml and various serum supplements. (—●—) 10% foetal calf serum, (—▲—) 5% foetal calf serum and 5% newborn calf serum, (—❍—) 5% foetal 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 (1981) 33,* by kind permission of the authors and publisher).

Different sera for various stages of the culture cycle

Since sera differ in their ability to assist attachment of cells and to promote proliferation (fig. 36) the best serum supplement will depend on the stage of the culture cycle. Sera active in promoting attachment of cells 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 is then important and sera providing this function 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 them changing to medium containing 5 % rabbit serum after 48-72 h (136). Foetal calf serum can be used to stimulate the growth of chicken embryo fibroblasts at low cell densities, whereas horse serum supplemented with 10 mM haemoglobin is more efficient for cultures at high cell densities (137).

Figure 37 illustrates that the use of foetal calf serum for the first three 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 foetal 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 this approach to providing a serum supplement is more economical than blending sera.

4.2.3 Variability of sera

One of the most important factors controlling the success and reproducibility of cell cultures is the variation between batches of a given type of serum (132, 138-141). 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 x 10^4 cells/ml with 3 mg Cytodex/ml) it is important to use a serum supplement of the highest possible quality.

Batches of a particular type of serum show large variations with respect to a number of components and this variation results in widely different plating efficiencies and yields, even if other culture conditions are optimal for growth (table 13). The batch of serum can also determine which components from the medium will become limiting. For example, the batch of serum is critical in determining the division-limiting concentrations of cystine (118) and variable levels of arginase can rapidly deplete the medium of arginine (130).

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 batches of serum. Since microcarrier culture is a method which is directed towards achieving the highest possible yields form a given volume of medium it is important to screen batches of serum whenever possible. Selection of the best batches of serum should be on the basis of plating tests in microcarrier culture. Batches of serum giving maximum cell attachment and growth in Petri dishes are not necessarily the most suitable batches for microcarrier culture (table 14). This effect may reflect different affinities the toxic components in the serum have for various culture surfaces.

Table 14. Effect of various batches of foetal 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 x 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. 1981, in press,* by kind permission of the authors and the publisher.)

Batches of sera can be screened by simple plating and growth tests on microcarriers contained in bacteriological Petri dishes. A simple test, modified from that described by *Federoff an Hall* (133), 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 14). In this test, medium containing 10% serum should be used. These tests can be performed in parallel with standard plating efficiency tests in monolayer culture (141).

Serum is a potential source of contamination and only batches free from microorganisms, including viruses, bacteriophage and mycoplasma, should be used.

4.2.4 Serum free media

The undefined nature of serum supplements and their variation in quality makes the use of serum-free media one of the goals of cell culture. Recent developments in formulating media (142) show that a wide variety of cells can be cultured in the absence of a serum supplement provided certain components are added to the medium. Certain formulations can support the growth of cells in microcarrier culture (143). The components which can be used to replace serum in microcarrier cutures include fibronectin, transferring, insulin and epidermal growth factor. Most serum-free media formulations are probably suitable for microcarrier culture but the stirred nature of this culture system means that additional, high molecular weight components such as serum albumin or Ficollâ are required in order to protect the cells (144). Soybean trypsin inhibitor (0.5 mg/ml) should be used when harvesting cells to be cultured in serum-free media.

4.3 Gas supply

Supply of correct amounts of O_2 and CO_2 is important to achieving high yields with microcarrier culture. Both O_2 and CO_2 have metabolic functions and CO_2 is also usually involved in the control of culture pH (section 4.4). The gas requirements of individual cells are the same whether grown in microcarrier culture or other systems. Unlike static culture 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 (76) observed the beneficial effects of medium movement on reducing microenvironments 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. Therefore examination alternative gas tensions can be valuable area when optimizing the microcarrier culture conditions.

4.3.1Gas 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 3.5, fig. 33). The gas in the headspace is renewed when taking samples or replenishing the medium (section 3.5.2). The headspace is briefly flushed with gas (15-20 sec of 95% air: 5 % CO_2 forced through a Pasteur pipette) before sealing the vessel. 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 a incubator having a humanized atmosphere (95% r.h.) with a constant supply of 95% air: 5 % CO_2 .

The exchange of gas by diffusion between the headspace and the culture is a relatively slow process and an important function of stirring microcarrier cultures is to improve this exchange. Because the recently modified vessels for stirred microcarrier cultures (section 3.2.2) operate at lower stirring speeds than the traditional spinner vesssels, the rate of gas exchange is reduced. It may 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 be taken to improve the supply of gas and they may also need to be considered when culturing primary cells or established cell lines, especially when culture densities exceed 2-3 x 10^6 cells/ml. These additional steps are not usually necessary for ormal diploid cell strains.

- A continuous supply of 95% air: CO_2 can be provided.
- Gas tensions in the culture headspace can be increased, e.g. by using gas mixtures with a higher concentration of O_2.
- Stirring speed can be increased. The increase can be between 25-50% but will depend on the cell type and the degree of confluence (section 3.5.1).
- The replenishment medium (section 3.5.2) can be gassed 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.
- Medium can be recirculated through a gas exchange vessel outside the culture (39).

These steps can also be used for improving the supply of gas in large scale microcarrier cultures. The purpose of such steps is usually to improve the supply of O_2 to cultures during the later stages of the culture cycle. These steps should be considered when cell yields are lower tha expected, when there is a sudden decline in growth rate or in combination with control of pH (section 4.4)

Note: Sparging of microcarrier cultures should be avoided. The erratic movement of gas bubbles can damage the cells and dislodge them from the microcarriers.

4.3.2 Oxygen

Oxygen is a key element for metabolism and the exact requirement for this gas in cell culture depends on the cell type, medium and stage of the culture cycle (145). 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 (145-151). In general, cultures should not be overgassed with O_2 since high tensions (above ambient) can be toxic and reduce growth rate. Higher tensions O_2 can be more toxic at alkaline culture pH (147).

Oxygen tensions affect proliferation rather than cell attachment. Under conditions of low culture densities, low tensions of O_2 (1-6%) are optimal for the growth of both normal diploid cell strains and established cell lines (147, 148). During the exponential growth phase the optimal tension of O_2 is usually slightly greater (148-152). 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% (149).

At the 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 10-12%) 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 conditions of low culture densities the medium should contain HEPES buffer to control pH (section 4.4.2).

When gas tensions can be monitored and controlled the most appropriate procedure is to start the culture with a low pO_2 (2-5%) and increase the tension during the culture cycle to about 15-20% at the end of the exponential phase of growth. This increase in pO_2 will also assist in controlling pH (section 4.4.2). The tension of pO_2 should be measured by immersing a suitable electrode in the medium.

4.3.3 Carbon dioxide

Solution of CO_2 in medium results in the formation of HCO_3^- an essential ion for the growth of 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 (153).

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 (153). This concentration was intended for studies on lung fibroblasts but has now become routine for general cell culture. The tension of CO_2, optimal for cell growth may be in the range of 0.5-2.0% with the exact value depending on cell type (153). To date most work with microcarrier cultures has involved CO_2, tensions of 5-10% 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 for control of gas tensions and pH with most large scale systems could be used to define the CO_2 tension which is optimal for growth.

Note: Leibovitz L-15 medium does not rely on CO_2 for buffering and control of pH and can be used when low tensions of CO_2 are required.

The role of CO_2 in control of pH is the most important aspect to consider when optimizing conditions for high cell yields and is discussed in section 4.4.

4.3.4 Purity of the gas supply

All gases used for cell culture should be of the highest possible quality. It is important that the gas supply is essentially free of CO, nitrous oxide and hydrocarbons (153). A membrane and/or cotton wool filter should be used to remove any particulate matter in gases which are introduced directly into cultures. Wide variations may exist in the actual pCO_2, levels of commercial gas mixtures (153) and by using certified sources this variable can be minimized.

4.4 Culture pH

Since pH influence 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 culture 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 4.4.2) or altering culture conditions such that the cells produce less lactate (section 4.4.3).

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.

4.4.1. pH optima for cell culture

In cell culture it is common to use a pH of 7.2-7.4 and the wide fluctuations in pH which often occur during the culture cycle and after medium replenishment (pH 7-8) have an adverse effect on cell yields (154). *Föhring et al* (54) concluded that a

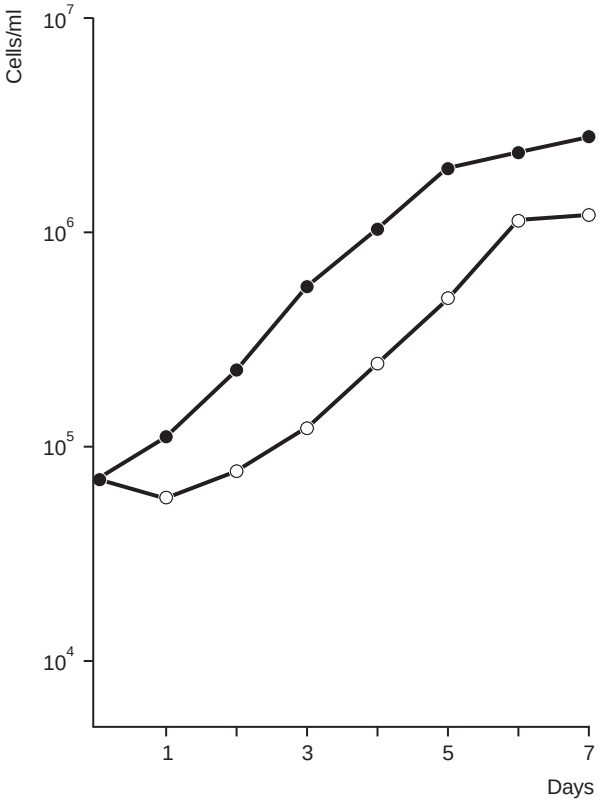


Fig. 38. Effect of pH on the plating effiency of cells. Plating was determined at 12–16 hr by ^{3}H- thymidine incorporation. (—●—) human embryo lung fibroblasts, (—❑—) SV40-transformed W138, (—○—) HeLa. (Ceccarini, C. *In Vitro 1 (1975) 78*, by kind permission of the authors and the publisher.)

constant pH was the most important parameter in determining growth rates and yields of cells and virus in microcarrier culture.

The attachment and plating efficiency of cells depends on the pH of the medium (fig. 38). One of the most critical stages of culture with respect to pH is just after inoculation (155) and in order 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. Therefore it is 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 3.4.1). HEPES buffer can also be used to ensure that the medium is not too alkaline during the early stages of the culture cycle (section 4.4.2). Diploid human fibroblasts are particularly sensitive to alkaline conditions (pH grater than 7.6) during the attachment stages of culture (fig. 38). When the 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 and then the pH can be increased to 7.6-7.8 for the exponential phase of growth. The pH should be decreased to 7.4-7.5 during the plateau stage of culture to ensure continued adhesion of the resting monolayers (section 3.5.3).

Table 15: The effect on pH on cell growth.

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

[a] pH measured at 25°C
[b] Relative to growth in bicarbonate-buffered medium, pH fluctuates from pH 8 to 7 after each refeeding.
[c] Essentially equal over indicated range.

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

The pH which is optimal for cell growth may not be the same as that which is optimal for cell attachment. Table 15 shows that by maintaining cultures at a pH optimal for cell growth substantial increases in yields can be achieved. Different types of cells may have different pH optima for growth (183, table 15). By maintaining the pH at a level optimal for growth it is possible to reduce the serum requirement by at least one half (155). 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 poH 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, 156). 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.

4.4.2 Buffers and the control of pH

The method used to monitor pH depends on the precision which is required. Good results with small scale culture (up to 1 liter) can be obtained simply by acting on changes in the colour of the phenol red indicator in the medium. In larger scale cultures it is normal to use electrodes to monitor pH. *McLimans* (104) describes how to control pH when preparing medium.

Most media utilize a CO_2/HCO_3^- buffer system but the capacity of this system is often not sufficient to be able to prevent a decreasing pH towards the end of the culture cycle. If a cell type produces large amounts of CO_2 then media based on Hank's salt solution (0.33 $NaHCO_3$/liter) are more suitable than media based on Earle's solution (2.20 g $NaHCO_3$/liter). Alternatively, a if cell type tends to produce large quantities of lactate then formulations with a higher concentration of HCO_3^- should be used. It is normal to use media containing Earle's salt solution in

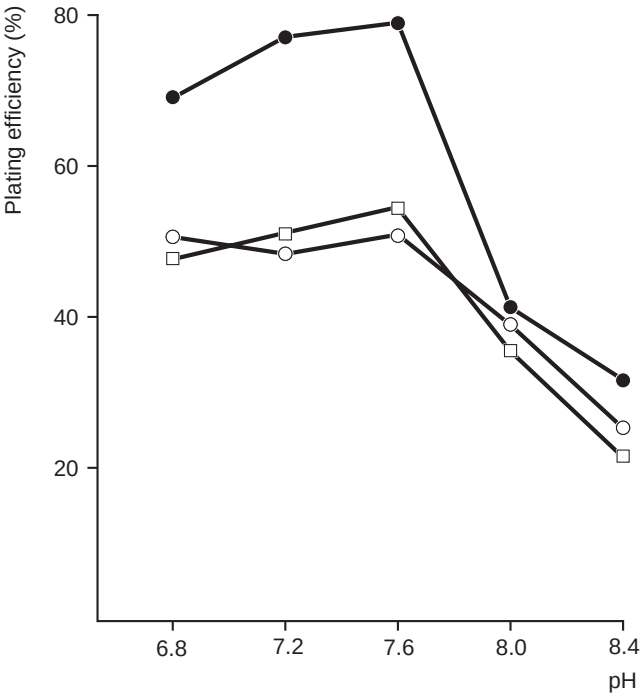


Fig. 39. Effect of control of pH on the growth of monkey kidney cells (Vero) in microcarrier cultures. Cultures (400 ml) were contained in sealed spinner vessels and stirred continuously at 50 rpm. Culture medium (DME, based on Earle's salt solution) was with (—●—) or without (—○—) 10 mM HEPES. The pH 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.)

combination with 95% air: 5% CO_2 and media containing Hank's salt solution with lower concentrations of CO_2 in the gas phase. Therefore microcarrier cultures contained in sealed spinner vessels usually require media based on Earle's salt solution.

Addition of HEPES delays the onset of pH drift and usually increases cell yield (fig. 39). The 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%) there is a lower stability of the HCO_3^- system and HEPES should be used. The exact amount of HEPES should be no more than is required to maintain the pH and 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 157, 158.

In large scale microcarrier cultures small changes in pH can be controlled by addition of HCO_3^- or increasing the tension of CO_2 and addition of NaOH or HCI can be used to control larger changes. The opportunity for constant monitoring and control with large scale systems means that HEPES is no longer essential for high cell yields. Culture pH can also be controlled when replenishing with fresh medium (section 3.5.2). Care should be taken not to change the osmolarity of the culture medium when adding buffers for pH control (section 4.5).

4.4.3 Minimizing accumulation of lactate

One of the most effective ways of avoiding difficulties with controlling a decline in pH is to use culture conditions which limit the formation of lactate. Some established cell lines produce large quantities of lactate and towards the end of the culture cycle when densities are high a rapid decrease in pH can occur even int the presence of addiotional buffer systems. This decrease in pH can result in reduced cell growth, viability and also detachment of cells from the microcarriers. Various methods can be used to limit production of lactate so that changes in pH are within the buffering capacity of the medium.

Cultured cells degrade glucose to either CO_2 or lactic acid and, depending on the redox state of the culture, high concentrations of glucose result in the formation of correspondingly high levels of lactate. When optimizing culture conditions an effective method for supplying glucose for obtaining maximum cell growth is to use 80 µM glucose for the initial stages of culture and to use daily refeeding with glucose to maintain a concentration of 25-40 µM in the presence of greater than 50 µM glutamine (124). This procedure reduce the production of lactate and encourages the use of glutamine as a source of carbon. Glutamine is a major source of energy and the degree of convention to lactate is less than when glucose is used as the energy source (124, 159, 160). A maintained or even increased supply of glutamine can also be used to reduce the formation of lactate (150). The amount of lactate secreted by transformed cells can be reduced by biotin (161) and replenishment media (e.g. table 12) can be supplemented with this component.

Zielke et al (124) described a medium which 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 hypoxantine (100 µM), glycine (100 µM), thymidine (40 µM) and uridine (100 µM).

An increased oxygen tension can be used to reduce the formation of lactate in the presence of glucose (146, 148). The oxygen tension can be increased during the culture cycle and can be used to encourage a more aerobic metabolism when culture density is high. Oxygen tensions of up to 20% can be used (section 4.3.2). Increasing the oxygen tension to approximately 15-20% of particularly useful for reducing lactate accumulation in stationary cultures of human fibroblasts (149).

Another method of reducing formation of lactate is to use a carbohydrate other than glucose in the culture medium. Fructose or galactose (2-10 mM) can be used instead of glucose and result in greatly reduced levels of lactate (159, 162). When using carbohydrates other than glucose it is important to maintain a good supply of glutamine since this component becomes a major source of energy (159). Glutamine

levels should be increased 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 x 10^4 cells/ml, with the minimum amount of medium.

Imamura et al (162) 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 approximately ¼ as muck lactate when using fructose or galactose instead of glucose as the carbon source (163).

Note: Leibovitz L-15 medium contains galactose instead of glucose and can be considered when experimenting with alternative carbon sources. The use of carbon sources and controlling accumulation of lactate should always be considered when optimizing medium replenishment schemes (section 3.5.2).

To summarize, useful procedures for avoiding a sudden fall in pH at the later stages of the 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 then alternative carbon sources should be used. 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.

4.5 Osmolarity

The growth and function of cells in culture depends on maintaining an appropriate osmolarity in the culture medium (164). 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 the osmolarity of the culture medium is kept within a narrow range. In the absence of evidence to the contrary the osmolarity of the medium used for the culture of any particular type of cell should be kept constant at a value in the range 280-320 mOsm/litre, normally 290-300 mOsm/litre. By controlling osmolarity it is possible to achieve more reproducible cultures.

Whenever the source of a particular culture medium is changed the osmolarity should be checked. Osmolarity of media produced by commercial suppliers may differ, probably because of differences in interpretation of original formulations (164).

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 (HCI), base (NaOH) and nutrients addition of NaCl and the correct amount required to achieve a particular osmolarity is calculated as follows (104).

The osmolarity of the medium is measured and the amount of stock NaCl (1 mg/ml) which must be added to achieve the desired osmolarity is calculated.

1 mg NaCl/ml = 1 ml stock (mOsm) = 32 mOsm increases.

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

where D (mOsm) = desired mOsm
O (mOsm) = observed mOsm
X = ml of stock NaCl (mOsm) to be added per milliliter of medium.

Measurement of osmolarity by freezing point depression is the most practical method (164). Dilution of nutrients in the medium by addition of large volumes of buffers or saline solutions should be avoided as much as possible.

4.6 Frezing cells for storage

4.6.1Procedure for freezing and thawing

The following procedures can be used for a wide variety of primary, normal and established cell cultures.

For the storage of cells as a suspension, exponentially growing cells are harvested from the microcarriers by the usual EDTA-trypsin or collagenase procedure (section 3.7.2) and resuspended (10^7 cells/ml) in storage medium (section 4.6.2). The cell suspension is centrifuged (400 g, 5 min, 4^{O}C), the supernatant is discarded and the pellet is gently resuspended in cool storage medium at a concentration of 3-5 x 10^6 cells/ml. One ml aliquots of the cell suspension are transferred to chilled sterile ampoules which are the cooled to -70^{O}C at -1^{O}C/min. This rate of cooling can be achieved by using a 5 cm-thick expanded-polystyrene box. The ampoules can then be transferred to the vapour or liquid phase of liquid nitrogen for long therm storage.

Cells may also be stored whilst still attached to the surface of Cytodex microcarriers. The cells to be stored are grown on the microcarriers until approximately 75 % confluent. The microcarriers are then rinsed in cool (4^{o}C) storage medium (section 4.6.2) containing one-half the final concetration of cryoprotectant e.g. glycerol or dimethylsulphoxide. After 5 min the supernatant is removed by gentle centrifugation and replaced by cool storage medium containing the final concentration of cryoprotectant. After a further 5 min the supernatant is removed and the microcarriers are resuspended in storage medium at a concentration of 5-10 x 10^6 cells/ml. Aliquots of this suspension are then stored as described above. During the washing steps at least 50 ml storage medium are required per g of microcarriers.

The additional washing steps for storage of cells attached to Cytodex microcarriers make sure that the microcarrier matrix is well penetrated with storage medium and thus breakage of the microcarriers during the freezing is prevented. Storage of cells on the Cytodex microcarriers has the advantage that harvesting is not required and cell viability is greater when the cells are frozen. Since the cells are already attached to the microcarriers, recovery after thawing is also improved. This can be particularly important for cells with a low plating efficiency where often less than 10 % of the cells in a stored suspension can attach to a cell culture surface and proliferate after thawing.

Recovery of frozen cells is achieved by rapidly thawing the ampoule in a 37^{O} C water bath and transferring the contents to a centrifuge tube. The cells and microcarriers are washed once in five volumes of growth medium, allowed to sediment and the supernatant is discarded. The cells and microcarriers are resuspended in fresh growth medium containing the usual serum supplement and transferred to the culture vessel for cultivation under standard conditions for 24 hr. After this incubation the microcarriers will be confluent and the cells can then be harvested in the usual way and used for further cultures (section 3.7). An advantage of using cells attached to microcarriers during the thawing step is that the storage medium can be easily removed from the cell preparation without the need for centrifugation.

4.6.2Storage medium

The most suitable storage medium is either 5 % (v/v) dimethylsulphoxide in growth medium or 10 % (v/v) glycerol in growth medium. The growth medium should contain a 10 % (v/v) heat-inactivated serum supplement. If glycerol is to be used in combination with freezing cells attached to cytodex microcarriers a higher concentration (20 %, v/v) should be used. If this concentration of glycerol cannot be used with a particular type of cell then the material should be rinsed at least one more time in storage medium containing 10 % (v/v) glycerol.

The cryoprotective agents should be of reagent grade and accumulation of oxidative products in the stock can be avoided by freezing ampoules of sterilized material. Dimethylsulphoxide 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 types of cells it can be desirable to use a mixture of 5 % (v/v) dimethylsuflphoxide in foetal calf serum.

When preparing the storage medium the cryoprotectant should be mixed well with the growth medium. The storage medium should be prepared immediately before use and should have a pH of 7.2-7.4.

4.7 Contamination

Prevention of 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 (165-167).

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. Therefore all routine cell culture in our laboratories at Pharmacia Fine Chemicals is performed in the absence of antibiotics.

Antibiotics can depress the growth rate and reduce longivity of animal cells in culture (168, 169). 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 in the absence of antibiotics for several passages (169). If microcarrier cultures are to be maintained at confluence for long periods of time the omission of antibiotics reduces any tendency for the cell layer to detach from established cell lines. Established cell lines may have been selected for growth in antibiotics and may be less sensitive than primary cells (170). Antibiotics can inhibit protein synthesis in primary cultures (170) and accumulate to high concentrations in fibroblast lysosomes (171).

In certain situations it may be necessary to use antibiotics. Antibiotics are required when working with primary cell cultures or cell lines suspected of being contaminated. The antibiotics can be withdrawn as soon as tests on the cells show them to be free from contamination. Antibiotics may also be necessary for culture systems were there may be a large risk of contaminating during the period of culture.

In large scale cultures the adverse effects of antibiotics are counteracted by the economical consequences of contamination. Therefore antibiotics are 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 sterilitui tests and if the medium is sterile when the culture approaches confluence, antibiotics can be withdrawn from the replenishment medium or at least the concentration can be reduced.

The most suitable antibiotics to use are penicillin (100 U/ml), streptomycin (100 µg/ml) or gentamycin (50 µg/ml) as antibacterial agents and nystatin (50 µg/ml) to eliminate growth of fungi and yeasts. These antibiotics can be used individually or in combination. The cytotoxicity of antibiotics increases in low-serum and serumfree media and the quantity of antibiotics should also be reduced in proportion to the serum concentration.

Cell cultures must be frequently checked for mycoplasma. Mycoplasma have a wide range of effects on cultured cell growth and function (172) and most importantly for microcarrier culture, mycoplasma compete with cells for nutrients in the culture medium. Mycoplasma contamination can result in rapid depletion of the essential amino acid arginine from the culture medum and an increased accumalation of ammonia (172). It is this depletion and imbalace in amino acid composition of the medium which has serious consequences for cell cultures. Extensive mycoplasma

infection usually leads to failure of the microcarrier culture. High density cultures of animal cells require all the nutrients available in the medium and can only suffer if they must compete with mycoplasma.

The most simple and effective method for routine screening for mycoplasma is to use the following fluorescent staining method (173, 174)

The cells to be tested are grown on glass coverslips in 5 cm Petri dishes until approximately 70 % confluent. Without removing all the culture medium, 2 ml of modified Carnoy´s fixative (3:1 absolute methanol: glacial acetic acid) are added gently to the dish containing the coverslips. 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 Hoexhst 33258 (American Hoechst, Somerville, NJ, USA or Riedel-De Haen AG, Seelze-Hannover, FDR) is prepared in HBSS without phenol red, pH 7.0. This solution is diluted to 0.05 µg/ml with HBSS and coverslip is washed 3 times in distilled water and mounted 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 and eliminate the mycoplasma (167, 172, 175).

5. Optimizing culture conditions and trouble shooting

Optimizing culture conditions

When microcarrier culture is to be used regularly for a particular type of cell it is advisable to optimize the culture procedures and conditions. Optimization is particularly important when microcarrier cultures are used for routine production of cell, viruses or cell products. The aim of optimization is to provide conditions which are suited to each type of cell and to each stage of the culture cycle. By using the most suitable conditions and procedures it is possible to maximize utilization of the microcarrier surface area and increase yields, to reduce the amount of cells needed for inoculation, to reduce the culture lag period, to improve reproducibility and to improve economy of serum and medium.

Optimization should take place in several stages and only after the basic culture procedure has been established. The steps can be summarized as follows.

1. **Control of inoculum condition**
   Routine work requires that the inoculum should be of consistent quality and have the highest possible plating efficiency (section 3.4.5). The cells should be checked routinely for the presence of mycoplasma (section 4.7).

2. **Modification of initial culture procedure**
   The static attachment period, initial culture volume and concentration of microcarriers should be adjusted so as to permit the highest possible plating efficiency (section 3.4).

3. **Adjusting stirring speeds**
   The stirring speed may need to be adjusted for each stage of the culture cycle so that there is maximum cell growth with no aggregation of the microcarriers (section 3.4.2, 3.5.1). Most optimal procedures involve a slight but progressive increase in stirring speed during the culture cycle.

4. **Defining a schedule for replenishment of medium**
   Replenishment medium can be used to slowly dilute the culture to the final volume. Such a procedure can be used to assist in control of pH and to prevent wide fluctuations in the concentration of nutrients (section 3.5.2). A continuous perfusion system can also be considered. The most important aspect of successful replenishment and obtaining high cell yields is to anticipate changes in pH and nutrient concentrations.

5. **Modifying the culture medium**
   The culture medium may need to be modified for certain stages of the culture cucle (section 3.4.6, 3.5.2, 4.1). The components of the medium should be altered if there are persistent difficulties in controlling pH (section 4.4.2, 4.4.3).

6. Reducing the requirement for serum supplements
Reducing the serum concentration and using different sera for different stages of the culture cycle should be investigated (section 4.2.2). Expensive supplements, e.g. foetal calf serum, usually only need to be present in the initial reduced culture volume; replenishment medium used for increasing the culture volume can often contain a reduced concentration of a cheaper type of serum (sections 3.5.2, 4.2.2) Batches of sera should be routinely screened with growth tests using microcarriers (section 4.2.3).

7. Modifying the gas supply
The gas tensions optimal for cell attachment and growth should be examined. A progressive increase in oxygen tenson during the culture cycle usually leads to improved control of pH and higher cell yields (sections 4.3.2, 4.4.3). The sueply of gas (95 % air: 5 % CO_2) should be used when possible to control pH.

8. Control of pH
The modification above (medium, gas supply) should also be considered with respect to control of pH. If possible the pH of the culture should be optimized for cell attachment, growth and maintenance of confluent monolayers (section 3.5.3, 4.4.1). When working regularly with cultures having a volume of one litre or more it is convenient to use a sterile pH electrode for monitoring the culture instead of acting on the basis of phenol red indicator.

Trouble shooting

When working with stirred microcarrier cultures for the first time some difficulties may be encountered. The following points list the occasional areas of difficulty and the most likely solutions. These points can also serve as a checklist when culturing each new type of cell.

1. Medium turns acid upon addition of microcarriers.
- Check that the microcarriers have been propberly prepared and hydrated (section 3.3)

2. Medium is alkaline at inoculation stage.
- Gas the culture vessel and equilibrate with 95 % air: 5 % CO_2 (section 3.4.1).

3. Loss of microcarriers on surface of culture vessel.
- Check that the culture vessel has been properly siliconized (section 3.2.4).

4. Poor attachment of cells and slow initial growth
- Ensure that the culture vessel is non-toxic and well washed after siliconization.
- Dilution of culture by PBS remaining after sterilization, rinse microcarriers in growth medium (section 3.3).
- Modify initial culture conditions, increase length of static attachment period, reduce initial culture volume or increase the size of inoculum (section 3.4).
- Control condition of inoculum (section 3.4.5).

- Eliminate vibration transmitted from stirring unit (section 3.2.1).
- Change to a more enriched medium for the initial culture phase (section 3.4.6).
- Check quality of serum supplement.
- 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 (section 3.4).
- Check that inoculation density is correct (3.4.4).
- Improve circulation of the microcarriers.

6. Aggregation of cells and microcarriers

- Modify initial culture conditions, reduce time that the culture is allowed to remain static(section 3.4.2)
- Increase stirring speed during growth phase (section 3.5.2), improve circulation of microcarriers.
- Reduce concentration of serum supplement as culture approaches confluence (section 4.2.2).
- Reduce concentration of Ca^{2+} and Mg^{2+} in the medium (section 3.5.2).

7. Rounded morphology of cells and poor flattering during growth phase

- Replenish medium (section 3.5.2).
- Check osmolarity (section 4.5) and pH (section 4.4) of culture medium.
- Reduce concentration of antibiotics if low concentrations of serum are beeing used (5 % or less).
- Check for contamiation by mycoplasma (section 4.7).

8. Rounding of cells when medium is changed

- Check temperature, pH and osmolarity of replenishment medium.
- Reduce serum concentration.

9. Cessation of growth during culture cycle

- Replenish medium (section 3.5.3) or change to a different medium (section 4.1.1).
- Check that pH is optimal for growth (section 4.4).
- Re-gas culture vessel or improve supply of gas (section 4.3).
- Reduce stirring speed (section 3.5.1).
- Check form contamination by mycoplasma (section 4.7).

10. Difficulties controlling pH

- Check that buffer system is appropriate (section 4.4.2).
- Improve supply of gas to culture vessel, lower concentration of CO_2 in headspace or increase supply of oxygen (section 4.3).
- Improve the supply of glutamine, supplement the medium with biotin or use an alternative carbon source, e.g galactose (section 4.4.3).

11. Difficulties maintaining confluent monolayers
    - Check that pH (section 4.3) and osmolarity (section 4.5) are optimal.
    - Reduce the concentration of the serum supplement (section 4.2.2).
    - Improve schedule for medium replenishment (section 3.5.3).
    - Reduce the concentrations of antibiotics (section 4.7).

12. Broken microcarriers
    - Ensure that dry microcarriers are handled carefully.
    - Check design of culture vessel/impeller and ensure that bearing is not immersed in culture (section 3.2.2).

6. Appendix

Cells cultured on Cytodex microcarriers

The purpose of these two lists is to allow rapid identification of cell types and also to recommend suitable inoculation densities and culture media. Table 17 provides a cross-referenc list of cell types, by tissue origin, which can be used to identify the specific cell lines in table 16. the lists do not include somatic cell hybrids which have been cultured on Cytodex microcarriers (e.g. figs. 6,7).

Table 16. Cell line specific list of cell types cultured on Cytodex microcarriers.

Cell	Species	Origin	Type[a]	Medium[b]	Inoc Density[c]	Comments[d]
A9	Mouse	Areolar fibroblast	E	1	L	1
Amniotic	Human	Amniotic fluid	P	2	H	2
BGM	Af. Green monkey	Kidney	E	3, 4	H	
BHK	Syrian hamster	Kidney	E	5	L	3
BSC-1	Af. Green monkey	Kidney	E	1, 2	H	20
Carcinoma	Human	Colon	E	1, 4	M	
Carcimona	Human	Squamous cell	E	4	H	
Carcimona	Human	Thyroid	E	1, 4		
Carcinoma	Rat (Lewis)	Lung	E	8	M	
Chang "D"	Human	Conjunctiva	E	21	H	4
Chang	Human	Liver	E	1, 4, 23	M	4
CHO	Chinese hamster	Ovary	E	1, 4, 8, 24	L	5
CR-1	Chimpanzee	Embryo lung	E	1, 12	M	
CV-1	Af. Green monkey	Kidney	E	1, 2, 4	H	
C6	Rat	Glial tumour	E	21	L	
Detroit 6	Human	Bone marrow	E	1, 4	L	4
Detroit 98	Human	Bone marrow	E	1, 4	L	4
Detroit 532	Human	Down's foreskin	ND	1, 8	H	6
Don	Chinese hamster	Lung	E	1, 2, 3, 11	H	
Endothelium	Bovine	Pulmonary artery	P	2	H	
Endothelium	Rabbit	Coronary artery	P	2	H	
Endothelium	Mouse	Brain capillary	P	2	H	
Endothelium	Human	Coronary artery	P	2	H	
EPC	Carp	Epithelioma	E	5, 13	H	7
FHM	Fat head minnow	Whole fish	E	5, 13	H	7
Fibroblast	Mouse	Embryo	P, ND	1, 2, 8, 12	H, M	24
Fibroblast	Chicken	Embryo	P, ND	17, 18, 19	H	24
Fibroblast	Rat	Embryo	P, ND	1, 2, 8, 12	H, M	24
Fibroblast	Rabbit	Embryo	ND	1, 2, 8, 12	H	24
Fibroblast	Human	Embryo	P, ND	1, 2, 8, 9	H, M	24
Fibroblast	Human	Xeroderma pigmentosum	P, ND	2, 11	H	8
Fibroblast	Muntjac	Adult skin	ND	21	M	
Fibrosarcoma	Mouse	Fibrosarcoma	E	4, 22	M	
Flow 2002	Human	Embryo lung	ND	1, 2, 8	M	25
Flow 4000	Human	Embryo kidney	ND	1, 2, 8	H	9, 24
FS-4	Human	Foreskin fibroblast	ND	1, 2, 8	H	10,24
Girardi heart	Human	Atrial appendage	E	1, 4	M	4
Glial	Rat	Brain	ND	1, 2, 8	H	
Glioma	Human	Brain tumour	E	1, 2, 8, 11	M	11
G1-V3	Af. Green monkey	Kidney	E	1, 12, 15	H	
HaK	Syrian hamster	Kidney	E	1, 8, 23	L	11

Cell	Species	Origin	Type[a]	Medium[b]	Inoc Density[c]	Comments[d]
HEL 299	Human	Embryo lung	ND	1, 2	H	
HeLa	Human	Cervical carcinoma	E	1, 4	M	
Hep2	Human	Larynx carcinoma	E	1, 8, 11, 16	L	4, 12
HTC	Rat	Morris hepatoma	E	1, 4, 11, 13	L	
HT 1080	Human	Fibrosarcoma	E	1, 8	M	13
IBR	Pig	Kidney	E	1, 8, 13	M	
IMR-90	Human	Lung fibroblast	ND	1, 2	H	24
Insect	Drosophila	Embryo	E	10, 25	H	14, 17
Insect	Trichoplusia	Ovaries	E	10, 25	H	14, 17
Insect	Spodoptera	Ovaries	E	10, 25	H	14
J 111	Human	Monocytic leukemia	E	1, 4, 11	M	4
KB	Human	Nasopharangeal carcinoma	E	13, 14	L	15
Kidney	Dog	Kidney	P, ND	1, 2	H	
Kidney	Rabbit	Kidney	P, ND	1, 2	H	
Kidney	Monkey	Kidney	P, ND	1, 2	H	
Kidney	Human	Kidney	ND	1, 2, 20	H	
Kidney	Bovine	Kidney	ND	1, 2	H	
Liver	Chimpanzee	Liver	E	1, 4, 12	L	
Liver	Human	Hepatocytes	P	2, 20	H	
Liver	Rat	Hepatocytes	E	2, 20	H	
Lung	Cat	Embryo lung	ND	1, 2	H	
LLC-RK_1	Rabbit	Kidney	E	1, 12, 15	M	16
LLC-RK_2	Rhesus monkey	Kidney	E	13, 15	M	
L-cells	Mouse	Areolar fibroblast	E	1, 8	L	
L-132	Human	Embryo lung	E	1, 20	M	
L-929	Mouse	Areolar fibroblast	E	1, 8	L	17
Lymphoblastoid	Human	Lymphoblastoid	E	2, 6, 15, 20	M	17
Lymphocytes	Human	peripheral blood	P	1, 6, 15, 20	H	17
Macrophage	Human	Peripheral blood	P	1, 6, 15, 20	H	18
Macrophage	Mouse	Peritoneal	P	1, 6, 15, 20	H	18
Macrophage	Mouse	Peripheral blood	P	1, 6, 15, 20	H	18
Macrophage	Rat	Peritoneal	P	1, 6, 15, 20	H	18
McCoy	Human	Sunovial fluid	E	1, 4	L	
MDBK	Bovine	Kidney	E	1, 8	M	
MDCK	Dog	Kidney	E	1, 3, 15	M	19
Melanoma	Dog	Melanoma	E	6	L	
Melanoma	Human	Melanoma	E	13	L	
Melanoma	Mouse	Melanotic tumour	E	2, 11, 22	H	25
Morris HM_1CM_1	Rat	Hepatoma	E	1, 8, 14	H	
MRC-5, MRC-9	Human	Embryo lung	ND	1, 2, 7	H	24
Muscle	Chicken	Myoblasts	P	1, 2, 17, 25	H	
Muscle	Rat	Mysclе-fibroblasts	E	4	L	
Mv 1 Lu	Mink	Lung	E	30	H	
NCTC-2544	Human	Skin epithelium	E	27	H	4, 20
Neuro-2a	Mouse	Neuroblastoma	E	1, 4	M	
NRK	Rat	Kidney	E	1, 8, 12	M	
NZ-white	Rabbit	Kidney	E	1, 2	M	
Osteosarcoma	Human	Bone tumour	E	1, 20	M	21
Pancreas	Rat	Pancreas	P	1, 2, 6	H	
Pituitary	Bovine	Pituitary	P, ND	1,28	H, M	
Pituiary	Human	Pituitary	P	2, 8, 16	H	
Pituitary	Rat	Pituitary	P	2, 8, 16	H	
PK-15	Pig	Kidney	E	1, 12	M	
Pt-K-1	Potoroo	Kidney	E	1, 2	H	

Cell	Species	Origin	Type[a]	Medium[b]	Inoc Density[c]	Comments[d]
PyY	Syrian hamster	Polyoma-BHK	E	5	L	22
P38801	Mouse	Macrophage	E	6	M	
RD	Human	Rhabdomyosarcoma	E	2	H	
RK-13	Rabbit	Kidney	E	1, 2 8	M	
RPMI 2650	Human	Nasal carcinoma	E	1	H	23
RTG	Rainbow trout	Gonad	E	5, 13	H	7
SC-1	Mouse	Embryo	E	1, 8	H	
SIRC	Rabbit	Cornea	E	1, 2, 8	H	
Tb 1 lu	Bat	Lung	ND,E?	30	H	
Thyroid	Pig	Thyroid	P, ND	29	H	
Vero	Af. Green monkey	Kidney	E	1, 3, 7, 8, 9	M	
WISH	Human	Amnion	E	1, 2	M	4
WI-38	Human	Embryo lung	ND	1, 2, 7	H	24
Y-1	Mouse	Adrenal cortex tumour	E	22	H	
3T3	Mouse	Embryo fibroblast	E	8	M	
3T6	Mouse	Embryo fibroblast	E	8	H	

Additional transformed cells	Type[a]	Medium[b]	Inoc. Density	Comments[a]
SV40 - Mouse fibroblast (3T3)	E	8, 12	L	
RSV - rat kidney(NRK)	E	8	L	
RSV - rat subcutaneous tumour (XC)	E	1, 8	M	
MSV - mouse fibroblast (3T3)	E	1, 8, 12	L	
SV40 - human fibroblast (WI-38)	E	1, 8, 12	M	
MSV - dog epithelial (doC1$_1$, MDCK)	E	1, 8, 12	M	

[a] type of cell line

P - Primary culture. Cells which have been dissociate from the tissue, usually by enzymes, and inoculated directly onto the microcarriers.

ND - Normal diploid cell line. These cells are normal in-so-far as they have the expected genetic complement, do not form tumours and have a finite lifespan in culture.

E - Established or transformed cell lines. Cell which can be cultured indefinitely. Such cells are often transformed in the sense that they can form tumours when injected into a suitable host animal. Many cell lines of this type are of tumour origin. Cell lines in this category are usually easier to grow than those in the two categories above.

[b]Culture media

For any particular type of cell several different media will prove satisfactory. The following list of media refers to successful culture of the above cell types on cytodex microcarriers. Media 1 and 2 are the most universally applicable media and unless there are indications to the contrary these two media are to be recommended for microcarrier culture. Media used for large scale microcarrier culture are often different from those listed below. In such cases, the expensive serum supplements are partially replaced by the cheaper embryo extracts or hydrolysates. Details of media can be found in sections 3, 4, 6, 3.5.2 and 4.1. Abbreviations are listed in section 6.4.

1. DME, NEAA, 5 %FCS, 5 % CS.
2. DME, NEAA, NUCS, 10 % FCS.
3. DME, 5 % FCS, 5 % CS.
4. MEEM, 10 % FCS.
5. Glasgow modification of Eagle's medium, 10 % CS, 10 % TPB.
6. RPMI 1640, 10 % FCS.
7. MEM, 7 % FCS 5 % CS, 3 % TPB, NEAA.
8. DME, 10 % FCS.
9. CMRL 1969, 10 % FCS.
10. TC100, 10 % FCS.
11. MEM, 20 % FCS.
12. DME, 5 % FCS.
13. MEM, 10 % CS.
14. MEM, 5 % CS, NEAA.
15. Medium 199, 5 % FCS.
16. DME, 20 % FCS.
17. Medium 199, 10 % FCS, 10 % TPB.
18. DME, 1 % CHS, 5 % CS, 10 % TPB.
19. F10, 2.5 % CHS, 20 % CS, 0,5 % BEE.
20. Medium 199, 10 % FCS.
21. F10, 10 % FCS or up to 20 % FCS.
22. F10, 15% HS, 2.5 % FCS.
23. BME, 10 % FCS.
24. F12, 10 % FCS.
25. Grace's medium, 10 % FCS, 0.5 % LH.
26. L-15, 10 % HS.
27. NCTC 135, 10 % HuS.
28. McCoy 5 A, 10 % FCS.
29. NCTC 109, 20 % CS.
30. MEM, NEAA, reduced bicarbonate (0.85 g/l), 10 % FCS.

[c] Inoculation density

Includes only approximate classification of cells according to their optimal inoculation densities. These densities refer to cultures containing 3 mg Cytodex per millilitre and which are stirred continuosly in the final culture volume. If the cells are not available in sufficient quantities to be able to use these inoculation densities, the procedures outlined in section 3.4 can be used to obtain good results with fewer cells.

L - Low inoculation density, 20-70x10^3 cells/ml.
M - Medium inoculation density, 70-100x10^3 cells/ml.
H - High inoculation density,100-300x10^3 cells/ml.

It is recommended that newcomers to this technique begin with inoculation densities at the upper end of each range

[d] Comments

1. Negative for hypoxanthine-guanine phosphoribosyl transferase. Resistant to thioguani and often used as a parent in somatic cellhybridization. Hybrids using this cell have also been cultured on Cytodex microcarriers.
2. Cultures must be started in a small volume, preferably 2-5 ml in wells or small dishes.
3. Morphology may become rounded during later stages of culture. Stirring speed should be reduced and steps taken to control pH (section 4.4).
4. Contaminated with HeLa.
5. Produces large quantity of lactate, pH shift can be delayed by 10-20 ml mM HEPES in medium.
6. From individual with Down's syndrome and therefore does not have normal diploid karyotype.
7. These cells should be grown at lower temperatures (25-30°C).
8. Mutant cells of this type are extremly difficult to grow in all culture systems. Care should be taken when harvesting and the period of exposure to trypsin should be as short as possible.

9. Very large cells and less than 100 cells/microcarrier are found at confluence. This cell is of interest for urokinase production.
10. These cells are often used for the production of HuIFNß.
11. Clumping of these cells can occur during later stages of microcarrier culture. This can be avoided by increasing the stirring speed slightly at the stage when the culture reaches confluence.
12. A very hardy cell line which can resist wide fluctuations in temperature, nutrition and environment without loss of viability.
13. Frequent renewal of medium (3 times/week) is essential for high cell yields.
14. Very careful techniques are required for these sensitive cells. Optimal culture conditions are different from those for mammalian cells.
15. Cultures can be started with as few as 10^4 cells/ml.
16. Cells should not be exposed to high levels of CO_2. Serum quality is a particularly important variable (section 4.2.3).
17. Some lines can grow in suspension without microcarriers. Such lines should be subcultured in static monolayer culture before microcarrier culture. Stirring speed may need to be reduced in order to make sure that such cells remain on the microcarriers.
18. Removal of these cells from culture surfaces is difficult (section 3.7.6). Experiments can often be performed with the cells remaining on the microcarriers.
19. May require high concentrations of trypsin for efficient removal from culture surfaces.
20. Frequent renewal of medium and control of pH to 7.3 is essential.
21. pH shift at later stages of culture should be controlled, otherwise cells detach. These cells produce large quantities of fibroblast interferon.
22. Stirring speed should be decreased during later stages of culture. High cell yields require HEPES as a buffer against lactate accumulation (section 4.4.2).
23. These cells grow as dense clusters. Faster stirring speeds may be required to avoid clumping and production of mucoid layers.
24. Good condition of the inoculum is essential (section 3.4.5) Cells should be taken from a growing rather than a stationary culture.
25. Rinsing culture with EDTA solution before trypsin is essential for rapid harvesting of cells (section 3.7.2).

Table 17. Tissue Specific list of cell types cultured on Cytodex microcarriers

Tissue	Cell line
Adrenal	Mouse cortex tumour — 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 thuroid
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
Fish	Rainbow trout gonad — RTG
	Fat head minnow — FHM
	Carp eptihelioma — EPC
Glial	Rat
Glial tumour	Rat — C6
Glioma	Human
Heart	Human atrial appendage — Girardi heart
Hepatoma	Rat — HTC, Morris MH_1C_1
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-MK_2
	African Green monkey — Vero, CV-1
	BSC-1, BGM, GL-V3
Leukemia	Human monocytic — J111

Tissue	Cell line
Liver	Human primary hepatocytes
	Rat primary hepatocytes
	Chimpanzee
	Human — Chang liver
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

6.2 Examples of microcarrier culture protocols

The protocols are signed for use with rod stirred cultures or recently modified spinner vessels (section 3.2.2). Cell numbers and volumes can be altered proportionately if smaller or larger culture volumes are required. The procedures should be followed after reading the information in sections 3 and 4.

6.2.1 Diploid human fibroblast and the production of interferon

The cells (MRC-5, WI-38, FS-4 etc.) should be used at as low a passage number as possible. Optimal results will be obtained when the cells are used between passages 10 and 25. The culture medium is described in table 12.

For a culture with a final volume of 500 ml, 2 g sterile Cytodex 1 and 100 ml of medium are added to the culture vessel. This concentrated mixture of microcarriers in inoculated with $6x10^7$ cells and incubated at 37°C. The cells are given sufficient time to attach and to begin to flatten on the microcarriers. This may take 6 h and during this period the culture should be stirred at 20 rpm for 2 min every 60 min. for those strains with a tendency to aggregate, slow (10 rpm) continuous stirring is required. Once the cells have flattened, the culture volume is increased to 250 ml with fresh medium and the culture is stirred at a speed just sufficient to keep all the microcarriers in suspension (20-30 rpm). After 2-3 days, 100 ml of medium is

discarded and the culture is diluted with fresh medium to 500 ml. Provided medium components do not become limiting, it is possible to achieve culture densities of $3x10^6$ cells/ml in 9-10 days. It is usually necessary to change some of the medium on day 5.

Interferon production
When the culture has reached confluence (8-10 d) the serum concentration is reduced to 2.5 % and the cells are cultured for a further 3 days. The culture medium is removed and the culture washed twice with 100 ml PBS and once with 100 ml DME. DME containing 5 mg/ml human plasma protein (or albumin) and 50 IU of fibroblast interferon/ml (optional) is added to 200 ml. The culture is gently stirred at 10-20 rpm. After 16 h the medium is removed and the culture is briefly washed in 50 ml DME containing DEAE-Dextran (100 µg/ml) 200 ml DME containing cycliheximide (10 µg/ml) and poly(I).poly(C) (20 g/ml) complexed with DEAE-Dextran ((100 µg/ml) are added and the culture is stirred gently at 10-20 rpm.

After 5 h actinomycin D (0.75 µg/ml) is added and gentle stirring is continued for 3 h. The medium is removed and the culture is washed three times in 50 ml DME with 10 min for each wash. 200 ml DME containing human or bovine albumin (Cohn Fract. V, 0.5 mg/ml), ascorbic acid (10^{-5} M) and pyruvate (10^{-3} M) are added and the culture is stirred at 10-20 rpm for 16-20 h and the culture fluid is collected as a source of crude interferon.

This procedure is based on those described in references 58 and 59.

Note: Better results can be obtained if antibiotics are not used and all solutions are prewarmed. The interferon production phase is carried out at 34°C.

6.2.2 African Green monkey kidney cells (Vero) and the production of Simian Virus 40

A culture medium based on DME supplemented with 10 % foetal calf serum is most suitable (table 12). For a 1 litre culture $1-5x10^7$ cells are added to 3 g of Cytodex 1 contained in 250 ml of medium. The culture is allowed to settle for 5-6 h with occasional stirring (20 rpm for 2 min every 60 min). If clumping of cells and microcarriers occurs, it may be necessary to stir continuously at 10 rpm. Once the cells have attached, the culture is diluted to 500 ml with fresh medium and stirring is commenced at a speed just sufficient to keep the microcarriers in suspension (approx. 20 rpm). After 1-2 days the culture is diluted to the final volume with fresh medium. At least 50 % of the culture medium should be replaced on about day 3-4. After 6-7 days the culture should contain $2-4x10^6$ cells/ml.

Virus production
The confluent microcarriers are washed twice in 100 ml medium without serum and are resuspended in medium containing 1 % (v/v) foetal calf serum. Serum levels above 5 % (v/v) often inhibit infection. The volume of medium should be just sufficient to cover the microcarriers. Virus stock is added at a MOI of approximately 10 PFU/cell. Cell number is determined prior to infection by the usual procedures (section 3.6.3). After adsorption at 37 ° for 2 h with occasional stirring,

the culture volume is increased to 500 ml with fresh medium containing 10 % (v/v) foetal calf serum. From this point on, the medium should contain a supplement of glucose (2 g/litre), non-essential amino acids and nucleosides (table 12 or section 6.4). Stirring should be just sufficient to keep the microcarriers in suspension (20-30 rpm). After 3-4 days the volume is increased to 1 litre with fresh medium and after 6-7 days a cytopathic effect is evident. At this point the medium can be collected or changed and collected 4 days later. During these later stages of culture, the volume can be decreased so as to increase the virus titre/ml. It is important that the pH should remain as close as possible to 7.2-7.4.

6.2.3 Primary monkey or dog kidney cells

This type of cell is often cultured on Cytodex 3 microcarriers for the production of large quantities of virus for vaccines. The kidneys from a single animal are briefly perfused via the renal vein with a solution of 0.25 % (W/v) trypsin and 0.1 % (W/v) collagenase in PBS at 37°C, pH 7.4. The tissue is then cut into small pieces with scalpels and after washing in cold PBS incubated at 4°C in Hanks' salt solution containing 0.025 M sodium citrate and 0.25 % (w/v) trypsin. After incubation for 20 h in 20-50 ml of this solution with gentle agitation the mixture is then warmed to 37°C and the tissues pieces are rinsed in medium containing serum (see below), resuspended in fresh medium and dispersed by a mixer. Debris is removed by passing the cell preparation through a 100 µm filter. Approximately 10^8 viable cells/ g wet weight of kidney can be obtained.

For a 1 litre culture approximately 4-6x10^7 cells are added to 4 g of Cytodex 1 suspended in 500 ml of culture medium. The culture is placed at 37°C and immediately stirred at a speed just sufficient to keep all the microcarriers in suspension (20-30 rpm). After about 12 h the culture is diluted to 1 litre with fresh medium. Provided medium componets do not become limiting during the later stages of culture, it is possible to achieve culture densities of greater than 2x10^6 cells/ml in about 7-8 days. It is usually necessary to change some of the medium on day 4; replacing 50 % of the volume with fresh medium is sufficient.

The final cell yield can be influenced by the age and source of the kidneys. For small scale cultures a suitable medium is MEM supplemented with 10 % (v/v) newborn calf serum. A more economical alternative for large scale cultures is MEM supplemented with 5-8 % (v/v) newborn calf serum and 0.25 % (w/v) lactalbumin hydrolysate.

6.2.4 Primary chicken embryo fibroblasts

A suitable culture medium for chicken embryo fibroblasts is DME supplemented with 1 % chicken serum, 5 % calf serum and 10 % tryptose phosphate broth.

Preparation of primary chick embryo fibroblasts

Embryos are removed aseptically from 9-11 day eggs and placed in a sterile Petri dish. The embryo is decapitated and transferred to a 50 ml centrifuge tube (plastic, conical). The embryo is minced briefly with a spatula, rinsed twice with 10 ml warm Ca^{2+}-free, Mg^{2+}-free PBS to remove erythrocytes and minced further with a fine spatula to 1 mm^3 pieces. To this minced tissue is added 10 ml of warm 0.25 % (w/v)

trypsin in Tris saline and the suspension is pipetted up and down 10 times in a wide mouth pipette. This dissociation should be as gentle as possible and is repeated after the mixture has been incubated for 5-10 min at 37°C. The mixture is allowed to settle, the supernatant is collected and trypsin activity is neutralized by adding an equal volume of cold medium containing serum. The remaining pieces of tissue are dissociated as above and the cell suspensions are combined. The debris is washed several times by pipetting up and down in warm Tris salin and collecting the supernatant suspension.

The final, pooled cell suspension is centrifuged (600 rpm, 10 min) and the pellet is gently resuspended in 20 ml culture medium containing serum. The yield from such an embryo is approximately 1-2x10^8viable cells.

The above procedure gives a suspension of cells from the whole embryo. Under usual culture conditions fibroblast cells predominate, especially in the secondary culture. If a less heterogeneous primary inoculum is required, the skin can be dissected from the entire embryo. This is easily done with forceps and scalpels and the skin can be minced into small pieces in a Petri dish with scalpels. The skin pieces are washed briefly in Ca^{2+} -, Mg^{2+}-free PBS and then dissociated as above. Material isolate from this source contains a greater proportion of fibroblasts.

A difficulty which is sometimes encountered with material from certain strains of chickens, is excessive aggregation. Isolate chicken embryo cells tend to be rather sticky and this can be reduced by using collagenase instead of trypsin. In many situations a mixture of 0.1 collagenase in Tris saline results in cell suspensions with a reduced tendency to aggregate. EDTA should be omitted when using collagenase for tissue dissociation. When dissociation is complete a small amount of EDTA solution is added to inhibit the collagenase.

Initiating the culture

For a 1 litre culture 3 g of sterilized Cytodex microcarriers are added to the culture vessel in approximately 300 ml of culture medium. The culture is inoculated with 2x10^8 cells (approximately equivalent to the number of cells isolated from one egg) and stirred at about 10 rpm for 3-6 hours. This gentle stirring allows rapid attachment of a high proportion of cells (greater than 85 %) without excessive aggregation of cells and microcarriers. After this period of slow stirring the speed is increased to a rate just sufficient to keep all the microcarriers in suspension. After 24 hours the culture volume is increased to 500 ml with fresh medium.

The above figure is representative for both primary and secondary cultures. Since these cells have finite lifespan the size of the inoculum must usually be increased when working with later subcultures.

After 2 days the culture volume is increased to 700 ml with fresh medium and the final volume (1 litre) is achieved with fresh medium on day 3. It is usually necessary to change some of the medium during the period of rapid cell growth. Thus for best results 50 % of the medium should be changed on day 4-5. Confluence is achieved after 7-8 days and at least 90 % of the microcarriers should be covered with cells. The yield is usually 2-3x10^6 cells/ml using this procedure.

6.2.5 Baby hamster kidney cells (BHK)

A Suitable culture medium is Glasgow modification of Eagle's medium supplemented with 10 % tryptose phosphate broth and 5-10 % calf serum.

For a one litre culture approximately 6-8x10^7 cells are inoculated into 250 ml of medium containing 3 g of Cytodex 1 or Cytodex 3. The culture is stirred gently at 20 rpm for 2 min every 60 min. After 3-4 h the culture is diluted to 500 ml with fresh medium and stirred continuously at a speed just sufficient to keep the microcarriers in suspension (20-30 rpm). The culture is diluted to one litre after 2 days and it is usual to replace at least 50 % of the medium with fresh medium on day 4. Confluence is achieved after 5-6 days and corresponds to a culture density of 4-5x10^6 cells/ml.

6.3 Methods for determining the protein and DNA content of cells grown on microcarriers

Protein content

A few millilitres of microcarrier culture suspension are removed and added to a suitable tube. The microcarriers are allowed to settle, the medium is discarded and the microcarriers are washed three times in PBS. 1 ml of fresh PBS is added and the mixture is sonicated until all the cells have been thoroughly disrupted. The microcarriers are sedimented by centrifugation and a 100µl sample of the supernatant is removed and protein concentration is determined by the method of *Lowry et al* (see ref. 178). Fresh microcarriers in PBS and treated as above can be used to provide a reference sample. If necessary an additional sample from the culture can be used to determine cell number (section 3.6.2).

Note: The denatured-collagen layer on Cytodex 3 microcarriers will contribute to the protein concentration when vigorous sonication is used.

DNA content

A few millilitres of microcarrier culture suspension are removed and added to a suitable tube. The microcarriers are allowed to settle, the medium is discarded and the microcarriers are washed with PBS. The supernatant is discarded and 1 ml of PBS containing Mithramycin (10µl/ml) and Mg $C1_2$ (15 mM) added. The mixture is sonicated until all the cells have been thourghly disrupted. The microcarriers are removed by centrifugation and the supernatant is measured in a fluorimeter (Ex. 440 nm; Em. 540 nm) with reference to standards containing 0.2-16.0 µg DNA/ml. This method is based on the technique described by *Hill and Whatley* (179). An additional sample from the culture can be used to determine cell number (section 3.6.3).

6.4 Abbreviations

BEE	Beef embryo extract
BSA	Bovine serum albumin
cAMP	Adenosine cyclic monophosphate
CIG	Cold insoluble globulin
CHS	Chicken serum
CS	Calf serum (newborn or donor)
DEAE	Diethylaminoethyl
DME	Dulbecco's modification of Eagle's medium
EDTA	Ethylenediamine tetraacetic acid
FCS	Foetal calf serum
HBSS	Hank's Balanced Salt Solution
HEPES	Hydroxyethylpiperazine ethane sulphonic acid
HS	Horse serum
HuS	Human serum
LH	Lactalbumin hydrolysate
MEM	Minimal Essential Medium
MOI	Multiplicity of infection
NEAA	Non-essential amino acids (glycine, alanine, aspartic acid, asparagine, glutamic acid, each 0.1 mM; proline, serine, both 0.2 mM)
NUCS	Nucleosides (adenosine, guanosine, cytidine, uridine, each 30 µM; thymidine, 10 µM)
PBS	Phosphate buffered saline
PFU	Plaque forming units
TPB	Tryptose phosphate broth.

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