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Mario Delgado Echeverry e Hijos Ltda.

Abogados Consultores – Marcas y Patentes

Bogotá D.C. - Colombia
Avda. 22 Park Way No. 37 – 31 Of. 202
Tels. (571) 2444096 – 3690624
Fax (571) 2442496 A.A. No. 6207
E-mail: mdeabogados@cablenet.co
mdelawof@cablenet.co
mdelawof@impstal.net.co

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No. 06-099780- -00008-0000

Fecha: 2012-03-06 15:40:00 Dep. 2020 DIR.NUEVASCR
Tra. 2 PATENTES Eve: 1 REGDEPOSITO
Act. 523 RESPUESTA Folios: 64

REFERENCIA: Expediente No. 06 – 99.780

**TÍTULO: "LÍNEA CELULAR VERO ADAPTADA PARA CRECER EN
SUSPENSIÓN"**

SOLICITANTE: ZELLTEK S.A.

**TRÁMITE: Respuesta a Concepto Técnico notificado mediante Oficio
No. 19133 del 15 de diciembre de 2011.**

Atentamente se dirige a usted **JAIME EDUARDO DELGADO VILLEGAS**, en mi condición de Apoderado de la sociedad **ZELLTEK S.A.**, dentro del término legal, presento respuesta a Concepto Técnico notificado mediante Oficio No. 19133 del 15 de diciembre de 2011.

1. DE LA SOLICITUD DE PATENTE.

Reivindicaciones

El Examinador Técnico de Patentes objeto el Capítulo Reivindicatorio porque:

"Las reivindicaciones 1 a 3 que hacen referencia a una línea celular la cual es caracterizada por estar adaptada a diferentes condiciones de crecimiento, lo anterior corresponde a un resultado a alcanzar, puesto que la reivindicación incluiría no sólo la

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mdelawof@cable.net.co
mdelawof@impstal.net.co

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solución propuesta por el solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado."

"La reivindicación 5 indica el uso que puede dársele a la línea celular reivindicada, el cual no es una característica técnica que defina a la invención. Se sugiere hacer la aclaración respectiva."

"El objeto de la reivindicación 6 que hace referencia a un procedimiento para que este adecuadamente caracterizado debe incluir todas y cada una de las condiciones y características específicas que se requieren para llegar a la formación de la línea celular, es así que se sugiere que dicha reivindicación incluya las características que se reclaman en las reivindicaciones 7 y 9."

"Las reivindicaciones 10 a 20 que hacen referencia a un procedimiento para producir virus, aunque involucran la presencia de una línea celular adaptada (sVEROp66) corresponden a un uso o a una aplicación de esta para a generación de virus ya conocidos en el estado de la técnica, es así que se sugiere al solicitante eliminar dichas reivindicaciones."

"Las reivindicaciones 20 y 21 definen el producto generado (virus y vacuna) por el procedimiento de obtención, lo anterior no es correcto puesto que dichos productos pueden caracterizarse por su naturaleza, composición, etc."

"Por lo anterior, el capítulo reivindicatorio no cumple con el requisito de claridad de acuerdo al art. 30 de la Decisión 486".

R/: Nuestro poderdante se permite manifestar lo siguiente:

De acuerdo a las sugerencias del Examinador Técnico de Patentes se procedió a modificar el capítulo reivindicatorio, donde:

- Se incluyo en la reivindicación 1 el número del depósito de la línea celular y que está adaptada para crecer en un medio de cultivo libre de proteínas

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mdelawof@cable.net.co
mdelawof@impsat.net.co

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y la suspensión comprende células aisladas en ausencia de agregados de células.

- Se incluyeron las características específicas del procedimiento para obtener la línea celular VERO.
- Se eliminaron las reivindicaciones 4, 5, 7, 8, 9, 12, 14 a 21, con el fin de que el capítulo reivindicatorio además cumpla con el requisito de **Unidad de Invención**.

Por lo expresado anteriormente, se presenta un **NUEVO CAPÍTULO REIVINDICATORIO** de la invención, de acuerdo a lo requerido por el Examinador Técnico de Patentes y el cual comprende **cinco (5) Nuevas Reivindicaciones** en dos folios, que cumplen con los requisitos de claridad exigidos por el art. 30 y de unidad de invención del art. 25 de la Decisión 486.

2. DEL ANÁLISIS DE PATENTABILIDAD.

Novedad.

De acuerdo al análisis del examen técnico, el Examinador concluye que la novedad se ve afectada por el documento **D1 (Citotechnology)**, donde expresa:

*"Las características esenciales de la línea celular reclamada en la reivindicación 1 habían sido divulgadas previamente en **D1**, el cual da a conocer cultivos de la línea celular VERO. Dicha línea celular es capaz de ser cultivada bajo condiciones de suspensión celular y en un medio libre de suero fetal bovino, así mismo **D1** revela que esta línea celular no requiere un transportador o carrier para su desarrollo (folio 125, abstract)."*

"En consecuencia, la reivindicación 1 no es nueva, según lo divulgado en el documento D1."

Las reivindicaciones dependientes, e, 5 y 6 no mencionan alguna característica que haga nueva a la composición de la reivindicación 1, por lo cual se considera que no son nuevas."

Nivel Inventivo.

El Examinador objeta el nivel inventivo para la reivindicación 3 con el documento **D1** y **D3** porque:

"La reivindicación 3 consiste en la línea celular VERO caracterizada porque dicha suspensión comprende células aisladas en ausencia de agregados de células".

"D1 divulga cultivos de la línea celular VERO. Dicha línea celular es capaz de ser cultivada bajo condiciones de suspensión celular y en un medio libre de suero fetal bovino, así mismo D1 revela que esta línea celular no requiere un transportador o carrier para su desarrollo puesto que se generan aglomerados de células con un tamaño que varía según las condiciones del medio."

"La diferencia entre la línea celular de la invención, definida en la reivindicación 3 y la línea celular divulgada en D1 es que las células son células aisladas y no se presentan en forma de agregados."

"Por otro lado, D3 hace referencia a una nueva línea celular derivada de MCDK la cual esta adaptada para crecer en suspensión, en ausencia de un carrier o un soporte de adherencia y en un medio de cultivo libre de suero fetal de bovino y proteínas."

"La diferencia entre la línea celular de la invención, definida en la reivindicación 3 y lo divulgado en D3 consiste en que la línea celular corresponde a la línea celular VERO."

R/: Con relación a las objeciones del Examinador de Patentes con respecto a la **novedad y nivel inventivo** de la invención, el solicitante desea resaltar lo siguiente:

Se desprende del Estado de la Técnica relacionado a los cultivos celulares que las suspensiones de células aisladas son preferidas a aquellas que presentan agregados de células, por diversas ventajas que las primeras presentan, especialmente en cultivos de altas densidades para la producción de virus. Así se puede apreciar en documentos tales como el documento titulado "Potential and problems of animal cells in suspension cultura" de **CHRISTIAN H. LEIST, HANS-PETER MEYER y ARMIN FIECHTER (1990)**, ver **ANEXO 1**, especialmente en la página 11, párrafo 3 y en la página 33, párrafo 3.

Además, **JACK LITWIN**, en el trabajo titulado "The growth of Yero cells in suspension as cell-aggregates in serum-free media" (1992) que se adjunta en el **ANEXO 2**, para dar cumplimiento a la solicitud de suministro de información de acuerdo al artículo 46 de la Decisión 486 de la Comisión de la Comunidad Andina, sugiere que el tamaño de agregados depende del medio de cultivo. También hace referencia a que células VERO de diversos orígenes tienen requerimientos de distintos medios de cultivo (párrafo 5, pág. 172).

Estas afirmaciones son conocidas por los técnicos en la materia, e implican que los cultivos celulares son extremadamente sensibles a cualquier modificación de las variables operativas, de los componentes de los medios y de la estirpe genética de las células. Por lo que la obtención de una determinada línea celular VERO, y en particular de la **línea de la presente solicitud de patente de invención de nuestro cliente**, depende del grado de adaptación a crecer en suspensión, del medio de cultivo, de los tiempos de adaptación y también depende de la genética celular. Todos estos, aspectos que quedan totalmente determinados en el **NUEVO CAPITULO REIVINDICATORIO**.

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Por otra parte no existe una teoría que pueda explicar la relación entre medios de cultivos y el tamaño de los agregados que forman una línea celular VERO en suspensión. Menos, aún, las sugerencias de **LITWIN** de ninguna manera podrían sugerir o insinuar la obtención de una línea celular VERO capaz de crecer en suspensión como células aisladas, en ausencia de agregados. Por otra parte no existen publicaciones previas a la prioridad de la **presente solicitud** que divulguen la **obtención de suspensiones de células VERO en ausencia de agregados**, a pesar del sinnúmero de ventajas que ello implica para su uso en la elaboración de vacunas. Con estos mismos argumentos consideramos que de ninguna manera podría usarse un antecedente que involucrara una línea celular distinta (como la MDCK del documento citado como **D3** por el Examinador) para atacar la **altura inventiva de la presente invención**. Ya que las líneas celulares MDCK presentan requerimientos diferentes de medios de cultivos y procedimientos que son también diferentes.

Más aún no puede decirse que la combinación de las anterioridades más cercanas mencionadas por el Examinador sugieran **la obtención de la línea celular VERO adaptada para crecer en suspensión**, en ausencia de materiales de soporte para su adherencia y en un medio de cultivo libre de suero fetal bovino que es la línea celular depositada en **German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Alemania)** bajo el nombre **sVERO p66** y cuyo número asignado por la Autoridad de Depósito internacional es **DSM ACC2791**, tal como ha quedado definida la invención en el **NUEVO CAPITULO REIVINDICATORIO**.

NOVEDAD Y ALTURA INVENTIVA

La nueva línea celular VERO de la **presente solicitud de patente de invención de nuestro cliente**, puede crecer en ausencia de SFB, sin proteínas y en una suspensión en forma de células aisladas, en ausencia de agregados de células. Esta línea celular puede ser usada en gran escala en la producción de vacunas en reactores de fermentación estándares, en ausencia de material de soporte tipo carriers. Las suspensiones de células aisladas presentan ventajas notorias frente a las suspensiones de agregados de células, por ejemplo mejora la eficiencia en la producción de virus que involucran reactores de fermentación de alta densidad (tal como se menciona en la página 4 de la memoria descriptiva). Más aún, las suspensiones de células individuales permiten medios homogéneos con viabilidad incrementada frente a suspensiones de agregados de células. Por otra parte el conteo de células es más seguro y por lo tanto es más sencillo llevar a cabo la infección a determinada multiplicidad de infección.

Estas razones nos permiten afirmar que la **presente solicitud de patente de invención**, tal como ha sido definida en el nuevo pliego reivindicatorio no solamente es **nueva**, pues no ha sido descrita con anterioridad, sino que presenta **altura inventiva**, ya que no se desprende de forma evidente del estado de la técnica. Nótese que la tecnología descrita en la presente solicitud presenta enormes ventajas a la hora de ser utilizada para la producción de virus.

Tales ventajas frente al estado del arte son obvias para un experto en la materia, ya que:

- No se requiere de microcarriers, por lo que los costos de producción son sensiblemente menores y la eficiencia del proceso mayor.

- No existen agregados de células, lo que mejora el rendimiento, pues las células que se encuentran en el interior de los agregados presentan problemas de incorporación de nutrientes y deficiencias en la infección viral, en el proceso de fabricación de vacunas. Estos problemas se dan por el fenómeno de difusión deficiente por la presencia de agregados.
- No es necesaria la separación y el procesamiento de los microcarriers y de los agregados, lo que ahorra pasos de proceso.
- La concentración celular en cultivos celulares es mayor, llegando a 10^7 . Frente a uno o dos órdenes menores en los cultivos tradicionales reportados en el estado del arte.

La invención definida por el **nuevo pliego reivindicatorio** no es anticipada por **LITWIN**, pues éste no enseña unas células VERO tales como las depositadas en **German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Alemania) bajo el nombre sVERO p66** y cuyo número asignado por la Autoridad de Depósito internacional es **DSM ACC2791**. Tampoco enseña, ni sugiere una suspensión de estas células que sean capaces de crecer en suspensión en ausencia de agregados y en ausencia de proteínas. Además en este trabajo se reconoce en la página 173 la desventaja de trabajar con agregados de células: "There is a disadvantage if the aggregates become too large and dense resulting in necrosis and cell death in the center".

Tal como ha sido divulgado en la solicitud original **la ausencia de agregados de la presente invención** se observó en cada uno de los ejemplos dados. Se adjuntan fotografías que no hacen más que confirmar y graficar tales observaciones. Puede observarse en las fotos adjuntas, correspondientes al ejemplo 1, y tomadas con un microscopio Olympus X150, 10X y cámaras digitales: Nokia, 3.2 mpx y Sony W150 8,0 mpx, que no son detectables agregados de células.

Un problema técnico que resuelve la **presente solicitud de patente de invención de nuestro cliente**, es el de lograr la línea celular VERO depositada en **German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Alemania) bajo el nombre sVERO p66** y cuyo número asignado por la Autoridad de Depósito internacional es **DSM ACC2791, que puede crecer en suspensión como células individuales**, libre de agregados celulares en un medio libre de SFB, **libre de proteínas y en ausencia de materiales de soporte** tipo carriers o microcarriers, útil para la producción de virus, constituyendo un nuevo sistema de producción de virus sumamente eficiente, que **no ha sido sugerido en el estado de la técnica previo ni se desprende** de la yuxtaposición de las anterioridades citadas en el examen de fondo.

Por lo tanto, queda claramente demostrado que existen diferencias sustanciales entre los documentos **D1, D2 y D3** con la **línea celular que se desea proteger en la presente solicitud de patente de invención de nuestro poderdante**; para lo cual pone a consideración del Examinador Técnico de Patentes: un **NUEVO CAPÍTULO REIVINDICATORIO** el cual incluye **cinco Reivindicaciones (5)** que cumplen con los requisitos de **NOVEDAD, NIVEL INVENTIVO Y APLICACIÓN INDUSTRIAL** exigidos por la Decisión 486 del Acuerdo de la Comisión de la Comunidad Andina; además, estamos dando respuesta al Concepto Técnico notificado mediante Oficio No. 19133 del 15 de diciembre de 2011.

Por lo anterior, solicito respetuosamente se proceda a **CONCEDER** la Patente de Invención titulada: **"LÍNEA CELULAR VERO ADAPTADA PARA CRECER EN SUSPENSIÓN"** a nombre de la sociedad **ZELLTEK S.A.**

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mdelawof@cable.net.co
mdelawof@impsat.net.co

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Atentamente,



JAIME EDUARDO DELGADO VILLEGAS

C.C. No. 19.270.955 Bogotá

T.P. No. 43.308 C.S.J.

Anexos:
Nuevas Reivindicaciones.
Anexos 1 y 2.

REIVINDICACIONES

1. Una línea celular VERO adaptada para crecer en suspensión, en ausencia de materiales de soporte para su adherencia y en un medio de cultivo libre de suero fetal bovino **CARACTERIZADA PORQUE** es la línea celular depositada en German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Alemania) bajo el nombre sVERO p66 y cuyo número asignado por la Autoridad de Depósito internacional es DSM ACC2791.
2. La línea celular VERO adaptada para crecer en suspensión, de la Reivindicación 1, **CARACTERIZADA PORQUE** la línea celular cual está adaptada para crecer en un medio de cultivo libre de proteínas y la suspensión comprende células aisladas en ausencia de agregados de células.
3. Un procedimiento para obtener la línea celular VERO adaptada para crecer en suspensión, en ausencia de materiales de soporte para su adherencia y en un medio de cultivo libre de suero fetal bovino de la Reivindicación 1, **CARACTERIZADO PORQUE** comprende los siguientes pasos:
 - a. adaptar la línea celular VERO de crecimiento en adherencia Vero E6 (pasage 26, Banco Argentino de Células) a un medio de cultivo con bajo contenido de suero fetal bovino mediante un progresivo ajuste del medio desde un medio de cultivo con una alta concentración de suero fetal bovino de entre 7 y 15 % hasta llegar a un medio de cultivo de baja concentración de suero fetal bovino de entre 0 y 3 %, preferentemente 2%;
 - b. aplicar la línea celular VERO obtenida en el paso "a." a un cultivo en suspensión con el mismo medio de cultivo de baja concentración de suero fetal bovino de entre 0 y 3 %, preferentemente 2% durante un tiempo de al menos 30 días

- para que dicha línea celular se adapte a crecer en suspensión en ausencia de materiales de soporte para su adherencia; y
- c. adaptar la línea celular obtenida en "b." para crecer en un medio libre de suero fetal bovino y libre de proteína, mediante centrifugación.
4. El procedimiento para obtener la línea celular VERO de la Reivindicación 3, **CARACTERIZADO PORQUE** se obtienen virus para formular vacunas, teniendo que:
- a. hacer crecer la línea celular VERO adaptada para crecer en suspensión, en ausencia de materiales de soporte para su adherencia hasta una concentración de células de entre $1 \cdot 10^5$ y $30 \cdot 10^6$ células por ml de medio de cultivo;
 - b. infectar la línea celular del paso "a" con dicho virus con una multiplicidad óptima de infección (m.o.i.) de entre 0.001 y 10; y
 - c. recoger la producción de virus producida durante la infección.
5. El procedimiento de la Reivindicación 4 **CARACTERIZADO PORQUE** dicho virus es seleccionado del conjunto comprendido por los virus de virus de la polio; virus de la estomatitis vesicular; virus influenza; virus papilloma; virus herpes simplex (HSV); 1 y 2.

Potential and problems of animal cells in suspension culture

Christian H. Leist¹, Hans-Peter Meyer² and Armin Fiechter

Department of Biotechnology, Swiss Federal Institute of Technology (ETH), ETH-Hönggerberg,
Zürich, Switzerland

(Received 30 November 1989; accepted 18 February 1990)

Integrated suspension culture; Cell recycling; Process development; Compact loop bioreactor; Process control; Animal cell line; Human melanoma cell line; High value biological

Introduction: Significance of in vitro culture of animal cells

The recent area of cell culture technology started about 40 years ago with the results of Earle and co-workers (Earle et al., 1951). They developed a culture medium promoting in vitro cultivation of animal cells. In the meantime, a lot of progress has been made in the areas of cell biology, working methods and mass cultivation systems for animal and human cells.

Correspondence to: A. Fiechter, Dept. of Biotechnology, Swiss Federal Institute of Technology (ETH), ETH-Hönggerberg, CH 8093 Zürich, Switzerland.

Present addresses: ¹ Ciba-Geigy AG, K-681.1.45, CH 4002 Basel, Switzerland. ² Lonza AG, COEN 3, CH 3930 Visp, Switzerland.

Abbreviations: CHO = Chinese hamster ovary; CMC = carboxymethyl cellulose; D = dilution rate (d^{-1}); EGF = epidermal growth factor; FCS = fetal calf serum; FGF = fibroblast growth factor; G_0 = gap phase 0: quiescent phase in cell cycle; G_1 = gap phase 1; G_2 = gap phase 2; HPI = hepatic proliferation inhibitor; IGF = insulin-like growth factor; M = mitosis; n = number of samples; pCO_2 = partial pressure of carbon dioxide (mbar); PDGF = platelet-derived growth factor; pO_2 = partial pressure of oxygen (mbar); PFK = phosphofructokinase; q_s = specific substrate uptake rate ($g\ g^{-1}\ d^{-1}$); q_p = specific product formation rate ($g\ g^{-1}\ d^{-1}$); R = recycling rate (-); S = synthesis phase; SG_0S = sensitive phase for the reentrance into the following S-phase; STS = stirrer tip speed ($m\ s^{-1}$); TGF = transforming growth factor; t-PA = tissue-type plasminogen activator; x = biomass dry-weight concentration ($g\ l^{-1}$); $Y_{x/s}$ = yield coefficient (biomass per substrate) ($g\ g^{-1}$); μ = specific growth rate (d^{-1}); μ_{max} = maximal specific growth rate (d^{-1}).

Animal cells can be grown adherent or suspended. An efficient advantage in the fields of adherent cultivation technology was the development of the microcarriers (Van Wezel, 1967). Microcarriers are small spheres (diameter: 50–20 000 μm) of differently coated surface, treated plastic or biological polymeric substances (for details see Table 5) serving as growth surface for the cells. Whilst their specific density can be adjusted to that of the environment, the spheres can be kept in suspension by very small power input. Therefore high cell densities up to 10^6 cells per ml at high product yields were reached soon with microcarrier cultures. Examples for previous industrial applications are production of viruses (Giard et al., 1977) and human interferon (Giard et al., 1979). To date an impressive number of different methods for surface enlargement have been developed such as systems of tubes and plates, membranes folded in different ways, and highly sophisticated perfusion systems consisting of porous ceramic bodies as growth-promoting surface (Lydersen et al., 1985; Tao et al., 1987). Perfusion systems provide a simplified medium exchange without special cell-separation devices. Nevertheless, some very important disadvantages cannot be overlooked. They are inhomogeneous and show gradients for all culture variables like substrate concentrations, pH or pO_2 . Furthermore, culture and growth parameters such as cell density are poorly determinable. Therefore, feasibility of adherent culture technology for research and development including process development and optimization is reduced.

Submerged culture systems however, can be easily maintained as homogeneously mixed system. Its process parameters can be analytically determined, a prerequisite for mathematical modelling of growth and metabolism. Different technical solutions for the mixing problem of submerged animal cell cultures have been developed and already summarized by McLimans and co-workers in 1957. Originally classical culture systems were applied for suspension culture of higher eukaryotic cells as formerly used for microbes. The efficiency of those well known culture systems for animal cell culture is questionable, because of the very high shear forces evolved. Therefore improved mixing systems are necessary.

Stability of genotype represents a further prerequisite for a production system basing on animal cell culture. Poorly defined and controlled variables of the culture system bear a dangerous pool of (unknown!) selection pressures on the cell population. The identification of the essential variables for a possible selection pressure is a general problem of process development. Because of the different properties of animal cells compared with microorganisms (see below) a different set of variables becomes relevant and therefore a different set of properties of the culture system and the culture conditions becomes essential. For instance, mixing time as an essential parameter in microbial culture becomes insignificant at animal cell culture because of its slower metabolic activity; however, shear forces rise in relevance for animal cell culture.

The great success of genetic engineering and mass culture of prokaryotes and fungi could make the future necessity of animal cell culture as production system questionable. However, for several reasons several biologically active proteins can only be produced at present by their natural producers or very familiar species. Insufficient product secretion and post-translational processing (special glycosyla-

TABLE 1
Examples for recent animal cell culture products

Plasminogen activators:	urokinase-type plasminogen activator tissue-type plasminogen activator recombinant plasminogen activator
Hepatitis B vaccine	
Superoxide dismutase factor VIII	
Atrial natriuretic factor	
Lymphokines:	interferons interleukins tumor necrosis factor colony stimulating factor
Erythropoietin	
Human growth hormone	
Epidermal growth factor	
Granulocyte stimulating factor	
Transfer factor	
Macrophage activating factor	
Recombinant human insulin	
Protein C	
Orthoclone	
AIDS diagnostics	
Antibodies:	polyclonal antibodies monoclonal antibodies chimeric antibodies chemically or radioactive-labelled antibodies

tions, carboxylations, phosphorylations and sulfatizations) make the use of prokaryotes or even yeasts impossible. The recent success in genetic engineering of animal cells resulted in new and potential products in abundance. They are produced in high quality and quantity by animal or plant cells with transfected human genes, often amplified to several thousand copies. Pharmacologically active proteins applied in diagnosis and therapy of human and animals therapy are especially of future interest. Some examples for recent animal cell culture products are given in Table 1 (see also Ratafia, 1987; Backer et al., 1988; Köhler and Milstein, 1975; Mizrahi, 1981; Bodo, 1981; Familetti et al., 1981; Klein and Ricketts, 1981; Van Damme and Billiau, 1981, Kaufman et al., 1986; Bollon et al., 1988; Weaver et al., 1988; Nilsson et al., 1988; Smiley et al., 1989; Handa-Corrigan, 1989; Ten Dijke and Iwata, 1989).

A important future application of cell, tissue and organ cultures is their use as in vitro bioassay systems for improved toxicology (Klausner, 1987) or, for instance for the application of recombinant human hormones (Bidey, 1988). Due to increasing political opposition, but also because of the increasing number of substances that need to be tested, alternatives to living animal tests are being sought in the pharmaceutical industry, and more recently also in the food and cosmetic industry. Exactly defined cultivation procedures for cell, tissue and organ culture, and the knowledge of their metabolism and growth kinetics are an absolute requirement for

the development of reliable test procedures. The present state of research in cell metabolism and growth kinetics but also in on-line analytics is still far from being sufficiently efficient to design a defined cultivation system. Nevertheless, substantial progress has been made with regard to the improvement of the metabolism and growth kinetics of some interesting cell lines such as human melanoma (Leist, 1988) and CHO (Kurano et al., 1990a, b).

Crucial aspects of animal cell culture

Each of the crucial aspects of animal cell culture discussed below bears some serious problems originating mainly from biological properties. These cell biology-related problems result in a further set of difficulties, the culture technical and methodological inconsistencies.

The biological properties and their related problems include:

Morphology: 10–100 times greater than microorganisms; no cell wall; high degree of differentiation (numerous organelles); susceptible cytoskeleton involved in stabilization, in metabolism and growth control.

Genome: bigger and more complex than prokaryotic genomes; less explored than that of microorganisms; instable genotypes.

Biochemistry: more complex metabolism and regulation; high number of intermediate metabolites and catabolites exerting control functions; necessity of growth and adherence factors.

Cell division: more complex cell cycle; up to now unknown regulations rather different from microorganisms; 10–100 times longer than microorganism generation times; complex, switch from normal cell cycle to differentiated states.

A sophisticated in vitro culture system has to meet the requirements of all these components and factors. The resulting extracellularly (physical, chemical and engineering) based problems are:

- selection of an implementation strategy of the culture system,
 - design of a mixing system,
 - aseptic operation,
 - identification and quantification of physical culture parameters (shear stress, temperature, viscosity),
 - identification and quantification of chemical culture parameters (pH, redox potential, oxygen concentration and metabolic intermediates/end-products),
 - measurement and control.
- deep knowledge of cell growth and regulation is central to Cell biology*

Cell division of higher eukaryotes

Fig. 1 shows the layout of the cell division of higher eukaryotic cells. The normal cell cycle (-M-G₁-S-G₂-M-) can be interrupted by entering a quiescent stage (G₀). G₀ is characterized by no mitogenic and an altered metabolic activity. Former publications defined G₀ also as the resting phase. Nutrient limitations (Schirr-

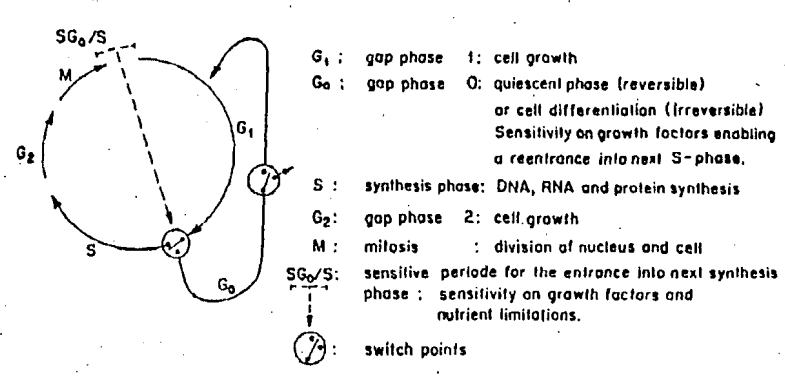


Fig. 1. Cell division of higher eukaryotic cells. The normal cell cycle (-M-G₁-S-G₂-M-) can be interrupted by reversible or irreversible entrance into the quiescent stage G₀ (see also Leist, 1988). Effectors of the switch-points are presently unknown.

macher, 1986), depletion of growth factors (Schirmmacher, 1986; Baserga and Surmacz, 1987) or shear stress (Croughan and Wang, 1989) can be responsible for this escape from the regular cycle. Optimization of the growth medium and the selection of a culture system preventing nutrient limitations even in the cellular microenvironment have great importance for the in vitro culture of cells. Contrary to former views, the sensitive period (SG₀/S in Fig. 1) for extracellular effectors is not represented by the late G₁-phase (Baserga and Surmacz, 1987), rather than in the following stages or at the end of mitosis (Traganos et al., 1987). Different behaviour during the G₀-phase can be observed. For certain cells (e.g., muscle and nerves) differentiation starts in G₀ (Schirmmacher, 1986). For these cells the entrance into the G₀-phase is irreversible. A reentrance into the S-phase of incompletely differentiated cells represents a cancerogenic transformation, a kind of 'partial dedifferentiation'. Other cells enter G₀ as a reversible quiescent period. Their reentrance into the S-phase is dependent on the presence of growth factors such as serum factors or mitogens (Schirmmacher, 1986; Baserga and Surmacz, 1987).

The proliferation rate of a cell population is regulated by the portion of cells remaining in the G₀-phase (Baserga and Surmacz, 1987). For Swiss 3T3 cells Brooks and Riddle (1988) have demonstrated an increasing cell cycle variability at decreased proliferation rate by the limited availability of growth factors. This behaviour is predicted by their transition probability model. In addition, Brooks and Riddle reported an increased failure rate of cytokinesis at low proliferation rates, leading initially to the appearance of binucleate cells. Such features may be responsible for the generation of cells with different degrees of polyploidy.

Growth factors activate genes summarized as a group of growth-regulated genes (structure genes) coding for proliferation-regulating factors. A further group of growth-regulating genes has also been identified (regulator genes). They are identical with proto-oncogenes and their regulator genes, often coding for the cells' own growth factors (Baserga and Surmacz, 1987). Products of growth-regulating genes

act positively or negatively on growth-regulated genes, being structure genes coding for the cells' own growth factors (Bohmann et al., 1987).

Hence, growth regulation of normally proliferating cells consists of cooperation of positive and negative regulatory factors. Their synthesis is controlled by regulator genes. Positive regulatory factors can be identical with the well known growth factors (epidermal growth factor, EGF; platelet-derived growth factor, PDGF; fibroblast growth factor, FGF; insulin-like growth factors, IGFs; transforming growth factor- α , TGF- α) often supplied to serum-free media of in vitro animal cell cultivations. Alternatively, TGF- β (Moses et al., 1987), the hepatic proliferation inhibitor (HPI; Huggett et al., 1987), and mammatatine, a tissue-specific growth inhibitor (Ervin et al., 1989) were identified as negatively regulating factors. Certain environmental influences are able to inhibit the activation of the TGF- β precursor. However, cell proliferation runs deregulated if the influence of a negatively regulating factor (e.g., membrane-bound TGF- β) is reduced.

Three statements on growth regulation are valid:

1. Effects of growth regulating factors (growth factors) resulting in a transitory stimulation of proliferation.
2. Effects on structural genes for growth regulating factors or their regulator genes result in a stable and to the next generation transmitted stimulation of proliferation, and transformation of the cell.
3. Transformed cells, also called established, infinite, or malignant cells differ from normal, primary cells explanted from an animal or human body by unlimited proliferation and/or a multitude of properties in their metabolism, regulation and morphology.

Primary cells and established cell lines

Cancer is observed more often in tissues with an intact potential for regeneration (liver, kidney or glandular tissue) than in tissues with differentiated cells, staying irreversibly in G_0 -phase. Cancer therefore develops predominantly in tissues with continuously proliferating cells such as epithelial or stem cells. Partial dedifferentiation combined with a reentrance into cell cycle represents the initiation of cancerogenesis (neoplastic tissue).

Mitogenic (cancerogenic) physical, chemical or biological environmental factors exert these cancerogenesis initiators. A transformation of the cell is stressed by disturbing the very sensitive equilibrium of positive and negative stimulating substances such as TGF- β deactivation, resulting in unlimited growth (Moses et al., 1987).

However, proliferation regulation alone (without dedifferentiation) also can be disturbed by the generation of mutations (cancerogenic substances, γ -irradiation, UV light etc.) and by spontaneous mutations of growth-regulated or growth-regulatory genes. Mutagenesis of regulatory genes of the cells' own proto-oncogenes possibly results in expression of these proto-oncogenes controlling predominantly the cell cycle (Schirrmacher, 1986; Kahn and Graf, 1986; Denhardt et al., 1986).

Integration of the genome from retroviruses into host DNA hinders growth regulation of the cell by the same mechanism. Virus proto-oncogenes originate most

probably from mammalian cells and are accidentally integrated into viruses. The sequences of proto-oncogenes of different origin are very conservative. Viruses can cause cancer (e.g., leukemia) but viral cancerogenic effects are not common in man.

Distortion of proliferation control can also be achieved by variation of the gene doses of growth-regulated or growth-regulatory genes if their expression is gene dose-controlled. Crossing-overs, other chromosomal rearrangements and non-disjunctions result in altered gene doses.

The behaviour of established and of cancer cells is similar as well as their formation. Therefore established cells are also called 'cancer like cells'. Nevertheless the differences between established or cancer cells generated by transformation and their non-transformed precursors (primary cells) are manifold like:

- altered chromosome number (aneuploid),
- different growth kinetics,
- ability to overcome the 70-generation-limit ('infinite' growth),
- no contact inhibition at adherent growth (multilayers possible),
- changed adherence properties,
- different cell shape,
- different organization of micro-filaments,
- different response to regulator molecules,
- different growth-factor requirements,
- synthetic potential for cell-inherent growth factors,
- increased specific glucose consumption by decoupling of respiratory chain,
- changed cAMP/cGMP-level,
- increased ability for agglutination by lectins,
- reduction of flexibility of plasma membrane,
- difficulties in intercellular communication,
- changes of antigenic determinants,
- ability for translocation.

This catalogue fits the properties of both the established and the cancer cells. To distinguish cancer cells from established cells, the following three differences appear typical:

- capacity to form metastases,
- ability to nidate,
- ability for vascularization.

Not each cell line expresses every property as given. Also the strength of expression can vary considerably. Human melanoma cells in culture show contact inhibition but cannot translocate (Leist, 1988).

Morphology of animal cells

Animal cells, because of their size, their big organelles and the missing cell wall are much more sensitive to mechanical and hydrodynamic shear forces than microorganisms (Fig. 2). Nevertheless, a wide range of tolerated intensities of shear forces caused by mixing and aeration are observed between different cell lines. Human melanoma cells in serum-containing medium (5% FCS) show stress effects at more than 1.5 m s^{-1} stirrer tip speed (STS), whereas CHO (Chinese hamster

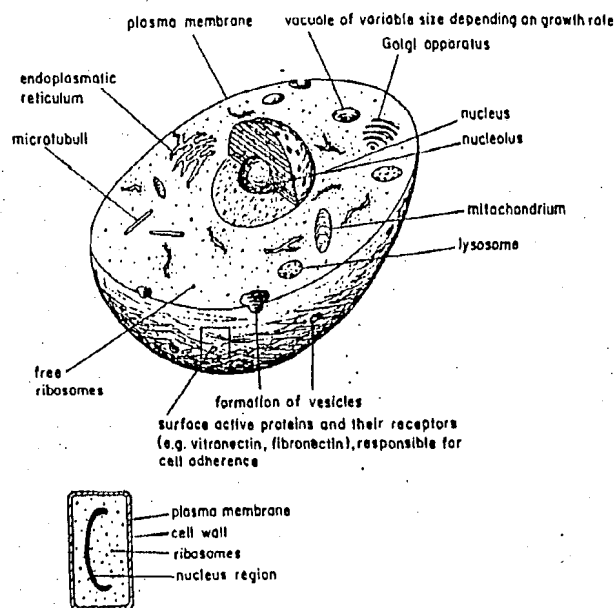


Fig. 2. Difference between animal cells and prokaryotes: Simplified cross-section through an animal cell and a non-phototropic bacterial cell (see also Leist, 1988).

ovary) cells in serum-containing medium (10% FCS) tolerate only 1 m s^{-1} Sparged aeration is not applicable for human melanoma cells because of their intolerance against gas bubbles, whereas hybridoma cells can be cultured in air-lift bioreactors (Bugarski et al., 1989). Also the mode of growth was seen to influence the limits of tolerated shear forces (Peterson et al., 1988). In suspension culture, pressure differences between stirrer front and backside, direct collision of cells with the stirrer or with baffles, especially turbulences and micro turbulences (micro eddies) are responsible for cell damage (see below). In adherent culture systems, hydrodynamic shear forces caused by medium flow (perfusion systems), pressure differences (perfusion systems), or direct collision of micro carriers per se or with the stirrer are observed.

In the case of strong accelerations, the big organelles may distort the cytoskeleton because of their inertia. Mechanical damage of the cytoskeleton normally results in cell death or at least in alterations of growth behaviour (Prescott et al., 1988).

The big cell diameter and the low surface to volume ratio are limiting factors of the internal mass transfer and uptake rate of nutrients. Thus the size of the cell also influences metabolic and growth velocity.

Control of metabolism

Mechanisms of metabolic control are quite well investigated for several micro-organisms and comprehensive models for metabolic pathways and control schedules

are available (Sonnleitner and Käppeli, 1986). For mammalian cells, however, metabolic pathways and regulatory systems from the very common mechanisms are known only incompletely (protein synthesis, for example, is often controlled by feedback mechanisms). The mechanisms of the growth factors effects have been investigated (Blok et al., 1988; Marshall et al., 1988; Miller et al., 1988a, b; Dean et al., 1988; Bascom et al., 1989; Band et al., 1989; Kojima et al., 1989; Warren and Pasternak, 1989). Some efforts have also been undertaken to analyse the basic metabolism, e.g., the glucose metabolism (Leist, 1988; Kurano et al., 1990b; Fiechter and Gmünder, 1989; Germinario et al., 1989) or the functions of glutamine, serving as amino acid and as C-source (Reitzer et al., 1979; McKeelhan, 1984; Bannai and Ishii, 1988; Kurano et al., 1990b). At present, the most coherent knowledge concerning animal cell metabolism is available for glucose metabolism. In quiescence and at low levels of metabolic activity, ATP production is running via respiration of free fatty acids and acetoacetate. At moderate performance, glucose is taken up by facilitated diffusion and serves as substrate. As long as the oxygen supply is sufficient and respiratory capacity is not exhausted, glucose is fully oxidized. At oxygen limitation and/or high energy transfer (decreasing ATP/ADP ratio), glycolytic activity increases. At saturation of the oxidative capacity, the reduction equivalents coming from glycolysis are transferred to pyruvate. Glycolysis is controlled by feedback regulation of the phosphofructokinase 1 (PFK 1) as described by the modified Sols-model for *Saccharomyces cerevisiae*. PFK 1 catalyses the conversion of fructose 6-phosphate to fructose 1,6-biphosphate and has therefore a key function in glycolytic control. PFK 1 is inhibited allosterically by ATP and citrate (by the latter especially in mammals and birds but lesser in yeasts), while it is activated by AMP, ADP and fructose 2,6-biphosphate. The metabolic pathways of transformed cells cannot be distinguished from those of normal cells by 'transformed' specific biochemical reactions, as it has been shown for micro-organisms in the case of interference by strong effectors. Transformed cells, however, can be distinguished from primary cells by the type of metabolic control.

Three not strictly independent explanations for the increased glucose utilization and lactate excretion of transformed cells are possible:

- Transformed cells show a higher glucose uptake rate compared to their respiratory capacity. The excess of glucose is metabolized to lactate, analogously to ethanol formation in *Saccharomyces cerevisiae* (Sonnleitner and Käppeli, 1986).
- An increase of glucose uptake is achieved by an increase in the number of transport proteins.
- Transformed cells manifest a decoupled regulation of the respiratory chain and thereby also a loss of control on lactate formation.

Fiechter and Gmünder (1989) presented an overview on the properties of metabolic control of tumor cells, in particular with regard to glucose degradation and suggest parallels with sugar metabolism control in glucose sensitive yeasts.

Genome and genetic stability

In contrast to the diploid genome of normal cells, cancer cells are aneuploid. Copy numbers (Brooks and Riddle, 1988) and even the type of chromosomes

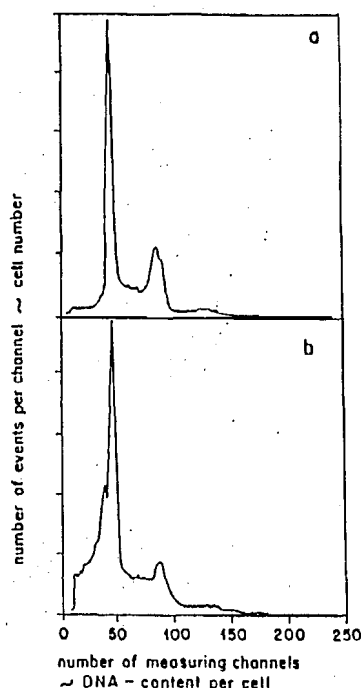


Fig. 3. Histograms of DNA distribution of two different human melanoma cell lines at identical growth conditions (T-flasks, 5% (v/v) FCS containing medium) determined by flow cytometry (see also Leist, 1988): (a) Cells of the cell line B4M16d: (1) peak (G_1): narrow and without side-peaks: genetical homogeneous population, no subpopulations. (2) Area beyond the shoulder and between the two peaks: normal S-phase portion (ca. 30%): normal proliferating population. (b) Cells of the cell line (clone) 84-12-26: (1) peak (G_1): a lot of side-peaks: each side-peak marks one genetically inhomogeneous subpopulation: (2) The relation of the area beyond the shoulder and between the two main peaks to the integral of the whole curve is much smaller than in a: in contrast to a, a DNA histogram of a slowly proliferating population is shown.

undergo variations in rather vast range, as has been shown for CHO cells with 19 to 24 different types. Also, for the aneuploid human melanoma cell line B4, a high genetic instability was demonstrated. Similarly, the cell line 84-12-26, a clone of B4, was shown by flow-cytometric DNA distribution analysis to be unstably aneuploid and to consist of a mixture of different subpopulations with varying amounts of DNA (Fig. 3b).

This inhomogeneity points to suboptimal culture conditions in submerged growth, resulting in selection effects and leading to inhomogeneous, genetically unstable populations. Therefore, a continuous growing cell line was established, well adapted to the conditions applied and kept stable in a chemostat with the aim to achieve a system free of undefined selection pressures.

A new serum- and protein-free growing cell line (B4M1 6d) was obtained with fully suspended constant growth and metabolic kinetics for 2 years (Leist, 1988). Also, excellent genetic stability and homogeneity (Fig. 3a) were demonstrated.

The culture medium represents therefore a potent tool for controlled and uncontrolled selection pressures. Depletion of some essential medium components forces genetic drift of a population towards self-production of this component (growth factors) or alterations of their metabolism (different C-source). The resulting population either appears as a well discernible subpopulation, or differs in only a few properties (e.g., specific product formation rate). Subpopulations of the latter type may be identified by a drastic decrease of volumetric product yield. Such reflections and investigations are essential for medium design and process development in all.

Culture methods

The bioreactor design for animal cell cultivation depends primarily on the mode of growth. It has to meet the requirement of the two principally different cell types growing fully submerged (suspended culture) or in adherent mode (surface dependent culture).

In suspension culture the cells are growing freely suspended in a liquid medium otherwise free of solids. In contrast, adherent cells are attached to a surface as a monolayer. Surfaces of synthetic material may be overlaid with liquid or solid media. Furthermore, two different modes of cultivation batch and continuous culture (or variations of them like repeated batch, fed batch and continuous with cell recycling) can be applied similar to microbial methods. Table 2 gives an overview on the bewildering array of techniques for the cultivation of microorganisms and higher eukaryotic cells. It shows the impressive progress of the last few decades in reactor and process design. As the underlying concepts are based on empirical approaches, the solutions given in the table are not conclusive in all aspects.

Suspension culture

Among all culture techniques, only suspension cultures can be homogeneously dispersed. This is a compulsory prerequisite for reproducible and defined culture conditions (particular of the micro-environment of each cell), reliable on-line analytics and for process control (see below).

Normally only malignant cells or established cell lines are able to grow in suspensions, but most of the industrially interesting cell lines such as CHO, BHK (baby hamster kidney), or hybridomas are established cell lines. Cell damage by agitation, mass transfer problems, formation of foam, floatation or sedimentation of cells, and other problems have to be compared with each other, and the most suitable cultivation system has to be evaluated. For each experiment readaptation of the population takes place irrespective of the type of bioreactor or media (Dittmar et al., 1984).

Originally rather simple systems such as dialysis tubes (Adamson et al., 1983), roller or shake flasks (Miltnerburger, 1977; Röder, 1968) were applied. One of the

TABLE 2
Overview on the cultivation technique available for microorganism and higher eukaryotic cells (see also Leist and Meussdoerffer, 1986)

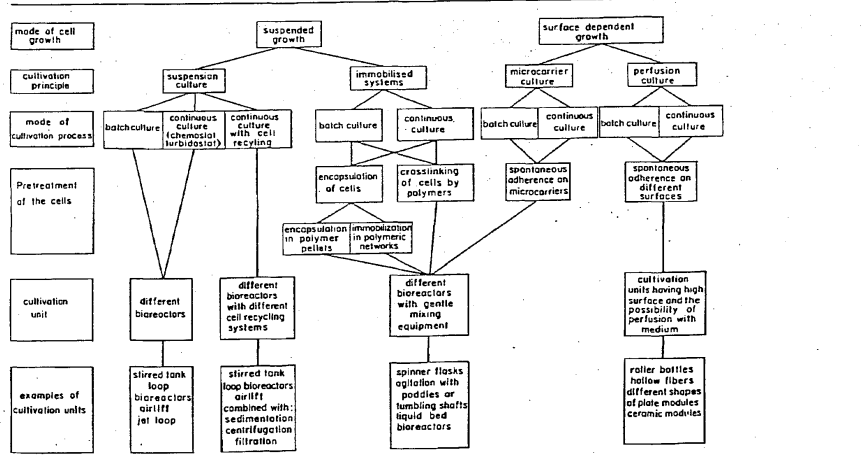


TABLE 3

A collection of agitation systems applied for cell culture. Low agitation speed stirrers are necessary for microcarrier or encapsulated cell cultures

<i>Mechanical agitation systems:</i>	
<i>Impellers</i>	Rushton type turbine (rather rare in cell culture) marine impeller in combination with a draft tube (Lynn and Acton, 1975; Miltenburger and David, 1980; Leist et al., 1986)
Paddle and coiled stirrer	
Vibromixer	(Lynn and Acton, 1975; Girard, 1977; Tolbert et al., 1982)
Agitated membranes	Agitation and aeration (Lehmann et al., 1985)
<i>Pneumatic agitation</i>	
Airlift	(Katinger and Scheirer, 1979; Katinger et al., 1979)

most frequent used units for animal cell culture is the magnetically stirred culture flask (spinner-flask). Turning cylinders with baffles have been developed for the cultivation of plant and animal cells. The systems were tested at different sets of culture conditions and compared with stirred tank bioreactors (Tanaka et al., 1983). All of these units satisfy lab scale requirements and have the advantage of simplicity and cheapness. For scale up, however, they are not at all suitable, except by designing a process consisting of several integrated small units. Such systems are time-consuming in maintenance, inhomogeneously mixed and therefore badly suited for process control. Hence, a single-unit-process in a well adapted bioreactor is required for scale-up.

Finally, the agitation-aeration principles applied in bench, pilot and industrial scale are of highest practical interest. The respective types for mechanical and pneumatic mixing are listed (Table 3).

The improvement of the reacting containment has essentially contributed to the continually growing list of new and highly valuable products as diagnostics and therapeutics synthesized by animal cells in suspension culture. An excerpt of the recent literature describing the prominent products is given below:

- lymphokines (Reuveny et al., 1980; Bodo, 1981; Pullen et al., 1985),
- monoclonal antibodies (Backer et al., 1988; Handa-Corrigan, 1989),
- growth factors (Witte et al., 1988),
- vaccines (Pay et al., 1985; Phillips et al., 1985).

Adherent culture

Because of their inhomogeneity with respect of gradients of all essential culture variables, adherent culture systems can only be optimized empirically. The determination and control of the culture parameters for all the cells is impossible and different undefined selection pressures, genetic drifts and subpopulations result. From the technical point of view it is clear that such systems cannot be managed continuously at a constant growth rate. Decoupling of growth and dilution rate, essential for a high volumetric productivity, cannot be achieved. From the ecological

TABLE 4
A collection of cultivation containments for adherent cell culture. The perfusion culture systems can also be applied for the cultivation for suspended growing mode

Roller bottles	
Thin-layer bioreactors:	rotating plates gyrogen bioreactors (Girard et al., 1979; Litwin, 1971; Moser, 1982)
Perfusion bioreactors:	immobilized bed fiber bed solid matrix (ceramic ("Opticell"), glass or steel wool) hollow fiber membranes (Lyderson et al., 1985; Spier and Whiteside, 1976; Varani et al., 1983; Whiteside and Spier, 1981; Bödeker, 1985; Griffiths et al., 1987; Familetti and Fredericks, 1988; Perry and Wang, 1989; Knight, 1989)
Immobilized systems:	microcarriers in suspension culture bioreactors microcarriers as immobilized or fluidized bed immobilized spheres of alginate or polyvinyl (Van Wezel, 1967; Girard et al., 1979; Pharmacia, 1981; Reuveny et al., 1983; Lai et al., 1984; Van Damme and Billiau, 1981; Prestidge et al., 1981; Familetti and Fredericks, 1988; Smiley et al., 1989; Griffiths et al., 1987; Nilsson et al., 1987; Iijima et al., 1988; Yamaji et al., 1989)

point of view apart from the much higher investment and maintenance costs, a loss of productivity has to be noted. A small remaining portion of the productive subpopulations and restricted utilization of substrates during a lowered production time reduce the volumetric productivity drastically (Table 9).

Nevertheless, primary and some established cell lines show surface-dependent growth. Cultivation on the wall of rotating, cylindric flasks (roller bottles) represents a standard method for animal cell culture. To date, a large number of different adherent culture methods has been developed. All these methods are based on the roller-bottle principle, partly with an enormously increased (support-) surface to (medium-) volume ratio. Table 4 shows a collection of surface-dependent cultivation systems demonstrating the disadvantages mentioned above. The advantages on the other hand are clear; a simplified medium exchange results and a complex cell-recycling system becomes unnecessary. Despite many classical immobilization techniques (Nilsson et al., 1987; Iijima et al., 1988; Yamaji et al., 1989) and recer. immobilized or perfusion production systems (Bödeker, 1985; Perry and Wang, 1989; Knight, 1989), only few dedicated substances are produced by adherent cultivation systems, mainly with cells grown on microcarriers (Van Damme and Billiau, 1981; Prestidge et al., 1981; Familetti and Nicoll, 1988; Smiley et al., 1989) or with cells immobilized in small spheres of biological or synthetic polymers (Familetti and Fredericks, 1988; Shirai et al., 1987, 1988; Bugarski et al., 1989).

TABLE 5
Different materials and surface laminations of available microcarriers are listed

Material	Surface lamination	Comment
DEAE-Sephadex A-50		Original microcarrier of van Wezel dextran matrix, diethylaminoethyl-groups → negative charge
Gelatin		
Collagen	Quarternary amine	Cytodex 1: homogeneous distribution of charge Cytodex 2: quarternary amine coated charged only on the surface
	Collagen	Cytodex 3: collagen coated
Cellulose		DEAE-cellulose
Glass		
Plastics: polystyrene		Surface charge
polyacrylamide		
polyacrolein	DEAE	
	Poly(D-lysine)	
	Diaminohexane	
	Gelatin	
	Collagen	
latex	Glass	

Both techniques try to combine advantages of suspended systems with those of adherent systems (Table 9). Homogeneity of the culture system for instance is possible with respect to the dispersion of the microcarriers. However, the inner surface of porous microcarriers and the microenvironment of microcarriers also represent regions with gradients for all culture conditions. Table 5 shows the different materials and surface laminations of microcarriers commercially available.

Batch versus continuous culture

Cyclic discontinuous (repeated batch) cultures (frequently referred as semi-continuous culture) are often applied for suspension culture. In a repeated batch culture 50–75% of the reaction mixture is replaced by fresh medium at the end of growth (compare also Fig. 8).

Suspension cultures are easy to maintain, as continuous systems (chemostat, turbidostat; Table 9). Nevertheless, only a few publications deal with chemostat cultivation for the production of animal cells (Ray et al., 1988; Harbour et al., 1988; Mano et al., 1987; Karkare et al., 1984; Van Wezel et al., 1984; Griffiths and Pirt, 1967; Moser and Vecchio, 1967).

Prokaryotes and yeasts were shown to produce proteins rarely at maximal growth speed (μ_{max}) (Meyer et al., 1985; Matin, 1981). Similar observations have been made with proteins of some higher eukaryotes (Gronow and Bliem, 1983; Obijeski, 1985). As a result the control of growth rates becomes decisive for a high production rate. True control of the growth rate, however, is possible only in a continuous culture.

Fig. 4 shows the x/D diagram of a chemostat culture of the human melanoma cell line B4 in serum and protein-free medium. μ_{max} is presumed to be 0.54 d^{-1}

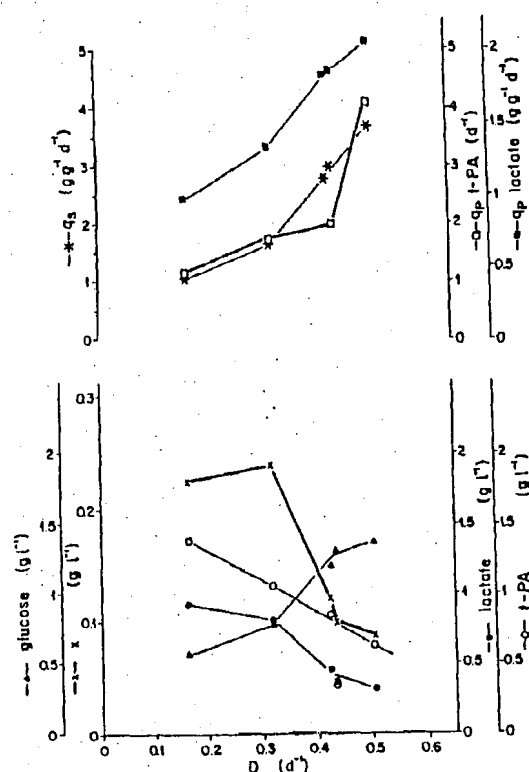


Fig. 4. x/D diagram of a chemostat culture of the human melanoma cell line B4 in serum- and protein-free defined medium. $\mu_{\max}$ is presumed at 0.54 d^{-1} (see also Leist, 1988). The occurrence of residual glucose at all D and the lower q_s at small D point to high apparent K_s .

(Leist, 1988). Thanks to medium development and a sophisticated process control (see below), the $\mu_{\max}$ from the serum-containing medium was attained in serum- and protein-free medium (Leist, 1988). The specific production rate (q_p) of tissue-type plasminogen activator (t-PA) shows a clear growth-rate dependence corresponding exactly to the q_p of lactate and the specific consumption rate (q_s) of glucose. t-PA produced by human melanoma cells is therefore one of the above-mentioned examples for the growth-related production of proteins. Despite the high q_p of t-PA, the absolute product concentrations in simple single-stage chemostat cultures are tremendously low. Responsible is the low biomass dry-weight concentration ($[x] = 0.25 \text{ g l}^{-1}$). Such product concentrations for high value products 10 to 100 are rather too low for industrial application.

These properties together with general slow growth rates ($0.001\text{--}0.05 \text{ h}^{-1}$) of higher eukaryotic cells (especially in serum- and protein-free media) compared to microorganisms in culture require an improvement of the cultivation system to increase productivity. The solution is given by decoupling μ from D for both types

of product synthesis, e.g., growth-related and not growth-related. The modification of the single-stage chemostat by cell recycling represents the only technical solution of the problem. On the contrary, to a single stage chemostat, its modification by cell recycling guarantees a high $[x]$ at all μ , even near $\mu_{\max}$ (important for growth-related products).

Continuous culture with biomass recycling

Besides sophisticated medium development and process control, the combination of chemostat technique with cell recycling requires a reliable (with respect to sterility, technical stability, and biological tolerance) continuous cell recycling system (filter, settler or continuous centrifugation). The cell recycling rate is free and independently eligible just as are μ and D corresponding to the equation: $\mu = D(1 - R)$. A steady state of this kind offers the possibility of real optimization with respect to the volumetric productivity and can only be achieved in suspension culture.

Cell recycling by cell sedimentation (cell settler) has the following advantages over centrifugation and filtration:

- technical reliability (no moving parts)
- low investment costs,
- low maintenance costs,
- easy sterilization and aseptic operation,
- no material toxicity problems,
- long-time stability (no fouling of components),
- easy and cheap scale-up.

Therefore, cell sedimentation is very suitable as a cell recycling method. For comparison and evaluation cell filtration is presently tested as an alternative method.

Fig. 5 shows an integrated continuous suspension culture system for higher eukaryotic cells. The system consists of a compact loop bioreactor combined with a cell recycling unit based on cell sedimentation. The system has been maintained with a human melanoma cell line in serum- and protein-free medium. The maximal cell density reached $2 \times 10^7 \text{ ml}^{-1}$ compared to $(3-5) \times 10^5 \text{ ml}^{-1}$ in chemostat culture without cell recycling. Over a period of several months $2 \times 10^6 \text{ ml}^{-1}$ cells and a viability of 95-99% (80% in chemostat) could be kept constant. This much higher viability is a direct result of the decoupling of μ and D , which guarantees a prevention from nutrient limitations or product inhibitions and provides therefore ideal growth rate and productivity.

Culture medium

Culture media for animal cells are much more complex and expensive than those for microbial culture. Some of the components are thermosensitive. This is crucial for sterilization and storage. None of the media on the market derive from systematic medium development (Barneš and Sato, 1980; Kawamoto et al., 1983; Ryan et al., 1987) as described for microorganisms (Kuhn et al., 1979; Goldberg

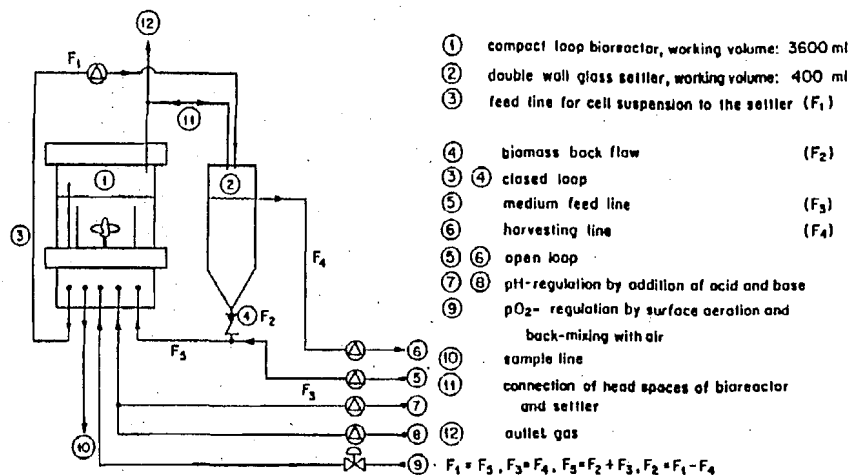


Fig. 5. Scheme of an integrated suspension culture system for the continuous cultivation of higher eukaryotic cells. Biomass is increased by continuous cell recycling based on cell sedimentation. The test system has been operated successfully for several weeks with a human melanoma cell line. The maximum cell density reached $2 \times 10^7 \text{ ml}^{-1}$ (see also Leist, 1988).

and Er-el, 1981). Often different literature references describe variations in the composition of the same medium. Therefore they are incomplete and/or too rich. Unknown limitations and sometimes inhibitions cause undefined selection pressure(s). This in turn results in genetic drift of the population up to an adaptation of the new population at the (undefined) culture conditions (Kawamoto et al., 1983) provided that these culture conditions are stable ("each medium creates its particular cell population"). At unstable conditions the selection process becomes infinite and the population is aimlessly drifting away.

As a result the exact adaptation of the medium to cell requirements and the attempt to keep culture conditions constant are the aims of each process development.

The complexity of the media is given by the high content of amino acids, vitamins and by the commonly added fetal sera. Serum is the most problematic medium component, because it:

- is the most expensive component,
- has variable composition from lot to lot,
- is chemically not defined,
- contains growth and metabolism inhibitors,
- hinders up stream processing due to foam formation and interference with filters, valves or pumps,
- hinders downstream processing by interference with products or down-stream equipment (filters, columns),
- represents the only stringent infection risk (viruses, mycoplasmas).

Despite these problems serum addition to culture media results also in a list of advantages as it:

- binds and neutralizes toxins,
- contains protease inhibitors,
- protects cells in agitated bioreactors from mechanical damage,
- increases buffering capacity,
- contains a complete set of growth factors, hormones and adherence factors.

To achieve these advantages however, serum can be substituted by chemically defined additives. The efforts necessary to identify the required additive(s) and the thereby altered culture condition(s) for a given production system (cell line and culture system) are very extended. As a consequence, serum is often added to culture media despite the disadvantages.

Commercially available standard media consist of six groups of components:

d-inorganic salts (incl. buffers and pH indicators).

d-energy source (e.g., glucose).

d-amino acids.

d-vitamins.

D-growth and adherence factors.

C-animal blood sera, or

d-polymers.

where: *d* = defined and cheap; *D* = defined but expensive; *C* = complex and expensive.

The function of sera and polymers

Cells of established cell lines are able to grow in serum free and chemically defined media (Barnes and Sato, 1980; Birch, 1980; Fischer and Wieser, 1983; Ham and McKeehan, 1979; Keay, 1979; Mitsuhashi, 1982; Sato, 1982), unless serum compensates for no hidden limitations (Birch and Cartwright, 1982). In the case of serum-free growth, the required growth factors, transport proteins and adherence factors are added to the medium or are produced by the cells themselves. The latter solution is possible because of the complete genetic potential of each cell in combination with changes in gene regulation pattern during transformation or later selection processes.

The high content of γ -globulins in blood serum of born animals possibly results in a decay of proliferation speed, and forces the use of fetal serum. Despite the use of fetal serum, concentrations of more than 10% are toxic or growth-inhibiting because of protease activities. *much less. via lact. or protease inhib.*

The high protein concentration results in a latent risk of foam formation even if applying gentle agitation and bubble-free aeration systems. Foam formation, however, is also temperature and salt-concentration dependent (Bumbullis and Schügerl, 1981; Bumbullis et al., 1979) and can therefore occur at even gentle agitation. *per*

Table 6 demonstrates clearly the serum dependence of the sensitivity of human melanoma cells to shear forces. Maximal tolerance for stirrer-tip speed increased by 50% with the addition of 5% (v/v) fetal calf serum (FCS). This effect is not based on an increase in apparent viscosity (Leist, 1988) but on a biological/biochemical

TABLE 6

Effect of FCS on growth behaviour of B4 cells and comparison of serum free growth behaviour of B4 and B4M16d cells

Cells	B4	B4	B4M16d
Number of samples	$n = 26$	$n = 69$	$n = 14$
Medium	RPMI	RPMI	RPMI
FCS	5%	—	—
CMC	—	0.1%	0.1%
Max. cell density (cells ml ⁻¹)	2.50×10^6	1.50×10^6	not determined
Viability (%)	95 ± 5	85 ± 5	95 ± 5
$\mu_{\max}$ (d ⁻¹)	0.52 ± 0.09	0.37 ± 0.15	0.54 ± 0.25
Max. lactate conc. (g l ⁻¹)	2.0	2.0	2.0
q_p for lactate (d ⁻¹)	1.42 ± 0.56	1.64 ± 0.40	2.79 ± 0.67
q_s for glucose (d ⁻¹)	1.92 ± 0.73	2.02 ± 0.50	3.16 ± 0.58

Temperature, pH and pO₂ are controlled, n represents the number of samples.

protection effect of the serum, most probably of serum proteins. With an increase of apparent viscosity in serum-free medium from 1 to 1.7 mPa by addition of 0.1% (w/v) carboxymethyl cellulose (CMC), the same rise in tolerated tip-speed was achieved (Leist, 1988). Other possible polymers for this purpose are polyvinylpyrrolidone, dextran, Ficoll 400 or polyglycol, but only CMC was tested for effects on growth of human melanoma cells. The effects of CMC on μ and viability of B4 cells grown in serum free medium are given in Fig. 6. A significant decline of $Y_{x/s}$ and viability at CMC concentrations of more than 3 g l⁻¹ is denoted. The slightly higher μ at 1 g l⁻¹ CMC compared with the control is remarkable. This effect agrees with the observations of Hamilton and Ham (1977) who described a stimulation of growth of CHO cells by 0.1% (w/v) CMC which acts by an unknown mechanism. Because of the protective effect without negative side effects CMC was added routinely to serum-free suspension cultures of human melanoma cells (Leist et al., 1988).

Despite the reduction in shear stress by CMC, a different growth pattern was observed in serum containing and serum-free medium. Table 6 (Leist, 1988) shows maximal cell densities and the mean values of the most important bio-parameters (q_p for lactate, q_s for glucose, μ , viability) of B4 and B4M16d cells in exponential growth phase of batch cultures in bioreactors.

The scattering of q_p and q_s is a result of different culture conditions and bioreactors used rather than a serum effect. Maximal lactate concentration is not only influenced by serum, but a tendency to an increase of q_p and also of q_s in serum-free conditions is noticeable. These differences may be explained by a 'stress' effect, generated by the omitted serum, containing some growth factors and transport or protection proteins. $\mu_{\max}$ and the resulting high maximal cell densities run low in serum free cultures for the same reason. Hence, a smaller part of the C-source is utilized for energy and biomass production, but a bigger one for lactate

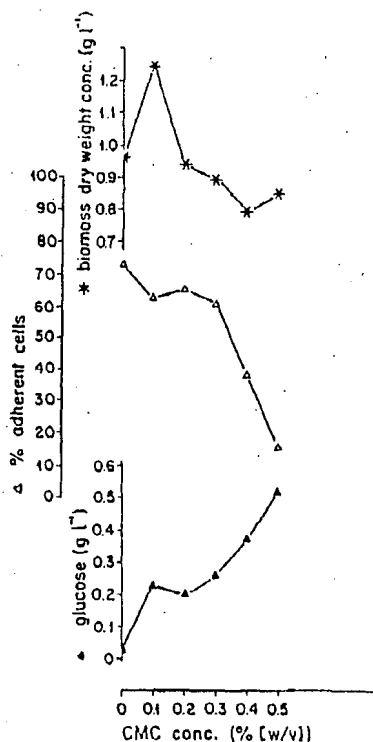


Fig. 6. Influence of CMC concentration on B4 cells grown for 7 d in serum free medium in T-flasks (see also Leist, 1988). Plots of biomass dry weight concentration, percentage of adherent cells and the residual glucose concentration at different CMC concentrations between 0 and 0.5% (w/v). With rising CMC concentration, biomass concentration and percentage of adherent cells decreases while residual glucose concentration increases. At 0.1% (w/v) CMC biomass concentration becomes even higher than at control (0% CMC).

formation. The reasons for this behaviour and its regulatory background are still unknown.

The reasons for the loss of viability in spite of CMC addition, however, must be different because protective effects of serum and polymers differ principally. The action of CMC is based on physical effects (increase of apparent viscosity), whereas serum acts on biochemical/biological levels (protective proteins). The two different protection mechanisms should manifest the morphological shape and/or composition of the cell membrane. Applying electron or light (confocal) microscopy, biochemical analytical methods and combinations of them (immuno fluorescence), characterization of these differences should be possible in future. Further effects of shear stress on metabolism and growth of human melanoma cells will be discussed later.

The lower μ_{max} and viability of B4 cells in serum-free medium point to a further function of the serum, namely to remove unknown limitations of incomplete media.

Despite a high q_s and q_p for lactate, B4M16d cells are well adapted to this serum-free incomplete medium. This is shown by high μ and viability. $\mu_{\max}$ of B4M16d in serum-free medium, however, is significantly higher than that of B4 cells in medium containing 5% FCS. In turn, this reference to reduced stress is caused by irreversible adaptation to suspended growth, the applied serum or protein-free medium. This, on the other hand, demonstrates the potential of biological adaptation by selection as an inverse and additive process to medium and process development (adaptation of biology $\langle - \rangle$ adaptation of environment).

Normally the ideal production system cannot be designed by one strategy alone. A mixture of both strategies as applied for human melanoma cells, offers the solution. Generally, process development has to be initiated by a catalogue of essential demands of the cellular, morphology and metabolism to technical, biochemical and physical properties of the culture system and to economical aspects of the production system. The priorities within this catalogue determine the character of the adaptation strategy applied.

Thermolability of medium components

Vitamins and a number of amino acids (e.g., glutamine and asparagine) are thermolabile. They represent a pool of chemical limitations or inhibitions by their catabolic products formed during storage or growth. Glutamine, for instance, is spontaneously degraded at 37°C by 50% within 6 d, resulting in 1 mol ammonia per mol glutamine (Kurano et al., 1990b; this issue). With this in mind, sterilization and storage of culture media have to be adapted accordingly. Sterilization by microfiltration can cause difficulties; e.g., serum-containing media are difficult to filtrate (Brock, 1983) and infection risks by viruses and mycoplasmas are high. Therefore, Nagle and Brown developed as early as in 1971 glutamine-free media for use in suspension cultures of different cell lines, allowing steam sterilization. Till now, however, not all thermolabile elements of the common cell culture media have been identified. Ultrafiltration could eliminate the infection risk but cannot be applied for all medium components (e.g., serum and proteins). Therefore only microfiltration is a reproducible sterilization technique also in large scale. To reduce the higher infection risk, two or more filtration steps can be connected in series. More important, however, is the evaluation and the strict complying with suitable handling procedures. During the 3 years of work with human melanoma cells, no infection of media has been observed (Leist, 1988).

Glutamine is an excellent example to demonstrate the role of thermolabile components of medium, because of its known chemical catabolic kinetics (Tritsch and Moore, 1962; Kurano et al., 1990b; this issue), as well as of its double effect upon the cellular metabolism. Glutamine serves not only as a C- and N-source, but also as energy source via oxidative processes (Arathoon and Telling, 1982; Kurano et al., 1990b; this issue). In the latter function glutamine competes with glucose and results in the same 'waste' products as lactate, pyruvate, and CO₂ (Brand et al., 1987). The results of Brand and co-workers demonstrate a higher rate of glutamine metabolism in proliferating rat thymocytes, than in quiescent (G₀) cells of the same origin. Even after prevention of heat degradation during sterilization the tempera-

ture-dependent spontaneous ring condensation of glutamine to pyrrolidonecarboxylic acid may occur. At 37°C, the whole glutamine content of the medium is degraded after a few days (Archibald, 1945; Griffiths and Pirt, 1967; Tritsch and Moore, 1962; Leist, 1988; Kurano et al., 1990b; this issue). Spontaneous degradation of glutamine fits to a negative exponential function and is temperature-dependent. At 4°C, the concentration still amounts to 70% of the initial value after 30 d, at 37°C, however, the 70% limit is already reached after 3 d, 50% after 6 d and 30% of the initial glutamine concentration after 10 d.

The following solutions for media preparation can be proposed:

- application of thermostable derivatives of glutamine such as glycyl L-glutamine, which is steam-resistant;
- cooling media to 0-4°C for storage; and
- separate continuous addition of the thermolabile components.

Water quality

The quality of tap or deionized water as used for microbial or fungal media is not adequate for cell culture. Normally bidistilled water is used to prevent infection with growth inhibitors. By additional heating of the bidistilled water used, further toxic substances passing both distillation steps will be removed (Leist, 1988). For the same reason all the material (tubes, needles, connections, filter cases and filters) have, after washing in detergent, to be rinsed and boiled (121°C for 30 min) in bidistilled water. Therefore special filter membranes (TC) withstanding steam sterilization and tubes of non-toxic materials (silicone or polypropylene) must be applied. Because of the formation of toxic products the medium should be protected from short wavelength light of the visible spectrum (Ham and McKeehan, 1979).

Culture parameters and process control

Besides the ability to maintain a homogeneously mixed system, reliable on-line data acquisition is required as a basis of efficient process control and control loops. Data acquisition in animal cell culture is still at the beginning, whereas data acquisition and process control in microbial cultures are now sophisticated (Sonnleitner and Fiechter, 1988; Sonnleitner, 1989). Even animal cells react on small and short deviations of some culture parameters such as temperature, pH or stirrer speed rather quickly (Leist et al., 1986). The on-line acquisition of data, however, is presently restricted to pH, temperature, pO₂, and pCO₂. There are several reasons for these shortcomings:

1. Handling methods, medium design, process development and process control of mammalian cell culture have been lagging behind over the last 50 years and develop only empirically and slowly;
2. Compared to microorganisms of interest (e.g., *E. coli* and yeasts) and despite some pioneer efforts (Dalili and Ollis, 1988; Ray et al., 1988; Glacken et al., 1988, 1989; Leist, 1988; Kurano et al., 1990a, b) metabolism and its regulation of animal cells are still much less investigated;

3. Essential biochemical and biological control parameters are more numerous for animal cell culture than for microbes;
4. Some of the biochemical and the biological parameters are cell-line specific. Data acquisition methods and control strategies have to be designed specifically for each process;
5. Most of the biological and biochemical parameters can only be measured by complicated off-line methods (enzymatic tests, HPLC, flow cytometry, antibody affinity etc.). Automatization and integration are therefore challenging tasks;
6. In situ on-line analysis for biological and biochemical parameters will depend on the application of biosensors (Karube, 1988). Problems of life time, stability and aseptical operation are still far from being solved. However, integration in a FIA-system (flow injection analysis = discontinuous on-line analytics in a bypass) is the immediate solution. FIA systems work outside sterile containment and therefore can easily be cleaned, repaired and calibrated;
7. Animal cells react to a variety of physical and chemical environmental conditions within very narrow limits. The tolerance range is often even smaller than the signal noise.

The variables to be controlled have to be identified and their optima and tolerance ranges have to be determined. For a human melanoma cell line this was successfully attempted with repeated batch culture (Leist et al., 1986). Investigation of further important factors such as glucose limitation or lactate inhibition were done with chemostat technique. Table 7 shows optima and tolerance ranges of human melanoma cells of some important parameters.

Reproducible and stable growth over several weeks in suspension was the criterion to define an optimum. For the definition of tolerance ranges, losses in μ , q_s , and viability were accepted. The very wide tolerance ranges were especially conspicuous for pO_2 and pCO_2 . Within these ranges, cells tolerated slow changes without measurable consequences. The temperature represents an exception. Low temperatures even for long periods are tolerated without irreversible consequences. Higher temperatures up to 37.5°C result in an immediate but reversible decay of growth (Leist et al., 1986). At temperatures of more than 38°C , however, the changes are irreversible. Sharp changes (within seconds) of all parameters, even within the tolerance ranges, have not been tolerated. Reversible significant decrease

TABLE 7

Optima and tolerance ranges of the most essential culture parameters of human melanoma cells grown in serum-free suspension culture

Parameter range	Optimum	Tolerance
pH	7.15-7.4	7.0-7.5
pO_2 (mbar)	40-160	20-200
pCO_2 (mbar)	0-200	< 250
Temperature ($^\circ\text{C}$)	37	?-37
Lactate conc. (g l^{-1})	-	0-3

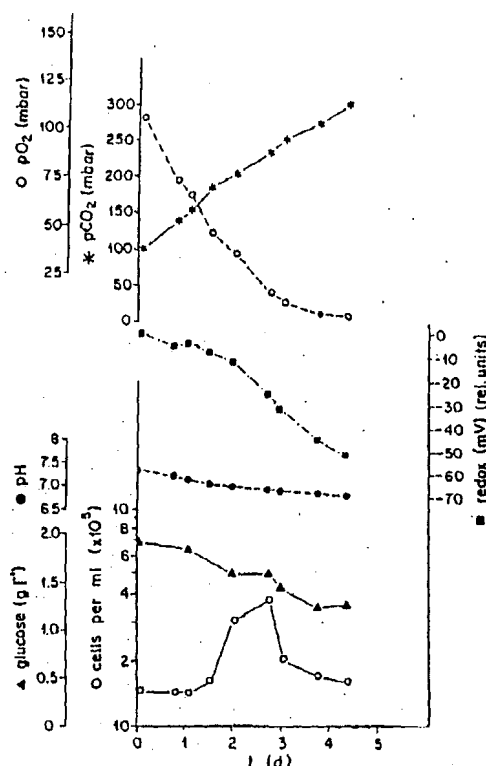


Fig. 7. Batch culture of B4 cells in serum containing medium (5% v/v FCS) in a 2 litre compact loop bioreactor with temperature control. As a relation of time (t) are shown: cell concentration and viability, residual glucose concentration, relative values of the redox potential, pH, pO_2 , and pCO_2 (see also Leist et al., 1986; Leist, 1988). All physical and chemical parameters show sharp fluctuations during growth, resulting in a long lag phase, short growth phase, low maximal cell density and early death phase.

in growth rate and increase in glucose metabolism became destroyed, indicating the occurrence of cellular stress (Leist, 1988).

Fig. 7 shows the course of batch culture without process control. This batch with B4 cells in serum-containing medium resulted in a growth stagnation at 4×10^5 cells per ml after as little as 1.5 d of growth, whereas a long lag phase (1.5 d) was observed. The death phase started after 3 d of culture. The pH decreased by 1 unit, CO_2 has been accumulated ($pCO_2 > 250$ mbar) and after 3 d the process became oxygen limited ($pO_2 < 5$ mbar).

Fig. 8 shows the results for repeated batch culture of B4M16d cells in serum-free medium with process control (temperature, pH, pO_2 , pCO_2). This figure represents any representative sequence of long-term culture and clearly demonstrates the potential of a simple but well adapted process control. Striking are the constant μ

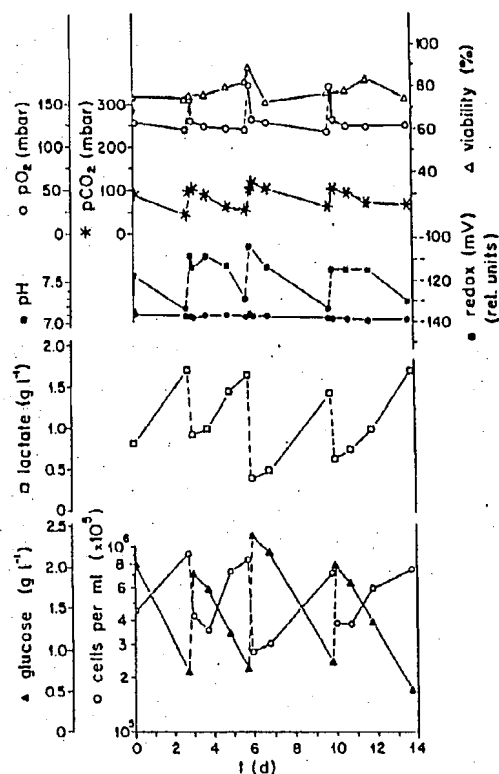


Fig. 8. Representative time period (2 weeks) of a repeated batch culture of B4 cells lasting all together 6 months. In contrast to the experiment of Fig. 7, a serum-free medium and control of pH, pO₂, and pCO₂ have been applied. As a result, short or no lag phases, stable growth and metabolism kinetics (identical slopes of biomass, glucose and lactate curves from one batch to the other), higher μ and viability as in Fig. 7 (see also Leist et al., 1986; Leist, 1988).

and q_5 from batch to batch. Despite serum-free growth and although the single batches were not cultured till stationary phase the maximal cell densities reached $((7-9) \times 10^5 \text{ cells ml}^{-1})$ were significantly higher than in Fig. 7. All measured values moved within the tolerance ranges. pO₂ for instance was managed within a 25 mbar range. The peaks of pO₂ at the beginning of each batch were caused by a too high pO₂ of the added fresh medium. The pCO₂ controlled indirectly by pH and pO₂ (Leist, 1988) was quite stable, therefore a control loop was unnecessary.

The non-periodical course of the redox signal could not be interpreted. The complex and changing composition of medium with progress of the cultivation and other influencing factors such as partial pressures of gases could be responsible for the observed effect. Therefore at the present the determination of this variable is not

useful in animal cell culture. The pH, however, seems to be important (Leist et al., 1986; Leist, 1988; Kurano et al., 1990a; this issue) and could be maintained within a narrow range of 0.05 units.

The on-line registration of biomass is one of the greatest recent analytical challenges. Some assays exist, all of them, however, are very cell or culture system specific or their signals show only poor correlation with cell density and express bad linearity. *Mei ap. de*

The currently available three approaches shall be presented: *—> comparison for de*

1. The on-line determination of an electrical potential of microorganism cell walls oxidized on platina electrodes seems to be very promising (Matsunaga et al., 1979, 1980).
2. An infrared sensor for on-line determination of hybridoma cell density (Merten et al., 1987). Its linearity, however, is restricted to cell densities between 1×10^5 and $2 \times 10^6 \text{ ml}^{-1}$ of cells and on a viability of more than 80%.
3. On-line fluorometry has successfully been applied for biomass determination in suspension cultures of different microorganisms (Meyer et al., 1984). Despite a good correlation of the fluorometer signal with biomass course, the results of on-line fluorometer detection in suspension cultures of B4 cells showed that only 13% of the signal was of cellular origin (i.e., cellular NADH) but 41% derived from extracellular products and 46% from the medium (Leist et al., 1986). The extracellular product was identified to be extracellular NADH. Additionally, the signal was found to be reduced by lactate (Leist, 1988). Comparison of fluorescence at different lactate and NADH concentrations demonstrated linear correlations of their concentrations with fluorescence intensity. These results explain the formerly observed sharp rises in the fluorometer signal during the first hours of the dying cultures (intact cells are broken down and release NADH, which is degraded after some hours) and the different correlation coefficients between cell density and fluorescence intensity (varying corresponding to actual lactate concentration).

Therefore this method is not suitable for on-line biomass determination, whereas further investigations should prove the fluorometer signal as a survey method for the biological activity and status of animal cells in suspension cultures.

Instead of continuous on-line analytical methods, discontinuous but rather fast off-line methods such as Coulter counter or flow cytometry could be applied. Both methods are partially suitable for automation, however, this increases complexity and costs. During cultivation of human melanoma cells flow cytometry was employed from time to time to determine the DNA content and the population diversity. Important hints, as for example the determination of population homogeneity, have been secured (Fig. 3). With biomass and DNA content determinations, however, the potential of this analytical method is far away from being exhausted. Alterations in DNA activity, of protein metabolism or of population dynamics can be registered within minutes. The possibility of having quick reactions and the changes in process management save a lot of time and money. On the contrary, traditional analytical methods such as viability determination are much too slow. For process or medium optimization the effects of different factors on the cells can *for de*

be characterized, therefore the flow cytometric analytical method should be established in each cell culture laboratory.

Long-term sterility of bioreactors

The most frequent origins of infections in animal cell culture are (arranged in order of decreasing frequency):

- handling errors,
- leakage at the peripheral equipment of the culture vessel,
- leakage at the culture vessel.

Aseptic operation, especially long-term sterility, is an important criterion for testing a culture system with respect to its suitability for mammalian cell culture. For the suspension culture of human melanoma cells carried out in compact short-loop bioreactors, the sources of infection were registered over a working period of 4 years. The results and observations are given in the following three sections.

Handling

Mistakes made during manipulations such as connection or disconnection of medium, inoculum, acid or base tubings represent the highest infection risk. Despite special equipment and sophisticated handling protocols, contaminations could not be excluded completely. Cleanness of the room greatly reduces the risk. After the working procedures had been improved, the infection probability was reduced to about 1% of all experiments performed. Cultivation systems could be kept sterile for more than 18 months (Leist, 1988).

Bacteria, fungi and yeasts were found as contaminants with the same probability, whereas cultures with bacterial infections, at least at starting infection, could be rescued with antibiotics. Despite on-line control of pH and pO_2 , routine control by microscopical inspection test remains important.

Culture vessels

The frequency and the source of infections caused by leaky culture vessels depends on the type of culture system. O-ring fittings represent the most frequent reason for infections in all culture systems. In suspension cultures with human melanoma cells in compact-loop bioreactors this source gave rise, depending on the culture time and environment of the bioreactor, to single or multiple infections with bacteria and fungi (Leist, 1988). Therefore all O-rings were routinely changed at the end of each culture.

Mechanical seals represent a further source of infection in mechanically agitated bioreactors. Using lab-scale bioreactors for the cultivation of human melanoma cells, no leakages have been noticed, even after 18 months of non-stop culture, with a single mechanical seal. The durability of the mechanical seals depends primarily on diameter, correct assembly and adequate lubrication. Therefore dual mechanical seals as often applied in lab- and pilot-scale bioreactors for mammalian cell cultures are not necessarily to warrant sterility.

Problems with heat stability of carrier or immobilization materials and inhomogeneity of heat distribution during sterilization represent a set of potential sources of infection in adherent culture systems instead of the mechanical seals.

Peripheral equipment of culture vessels

O-rings represent the highest infection risk not only for bioreactors themselves but also for their peripheral equipment. They are easily damaged during assembly or by heat. To handle (connect, disconnect) liquid-filled tubes or parts of the bioreactor is specially dangerous. Inlet and outlet gas treatment and the environment of the bioreactor are also very important factors. The infection frequency increased by a factor of 10 when the bioreactor for the cultivation of a human melanoma cell line was brought to a room containing several cultures of different microorganisms (Leist, 1988).

Infections in medium feed vessels caused by damaged stoppers can be prevented by appropriate cleaning and sealing.

The application of filter sterilization is absolutely non-problematic for medium preparation and for inlet/outlet gas processing. The periodical cleaning, sterilization and replacement of filter units for medium sterilization and the application of two filters in series for inlet and outlet gas processing result in an efficient reduction of infection risk (Leist, 1988).

Effects of agitation

Different damaging effects of agitation and aeration on cultured cells can be distinguished and are summarized in the following problem list:

1. *Direct collisions of the cells with the stirrers or baffles:* These effects can be reduced by a streamlined design of stirrer blades and baffles and by a reduced agitation rate. The latter solution, however, is limited by the necessity of a homogeneous mixing; an agitation rate that is too low results in cell sedimentation.
2. *Pressure differences between stirrer front and back side:* Lower slope of the stirrer blades, and lower agitation rate will reduce this effect. However, the necessity for homogeneous mixing restricts the lower limits for these reductions.
3. *Turbulences mediated by the differences in vector length of the vector field of non-laminar flow (degree of turbulence; Kolmogoroff, 1941):* Laminar agitation without turbulences, achieved by streamlined stirrers, would represent a hypothetical solution for this problem. However, other difficulties like inadequate mass transport would result.
4. *Micro eddies with a mean diameter smaller than the cellular diameter:* The size of micro eddies and their decay are affected by apparent viscosity (Croughan et al., 1987). An increase in apparent viscosity results in an expansion of the micro eddies diameter. When it exceeds the diameter of the cells (or that of cell-micro-carrier-particles) micro eddies lose their destructive effects on cells.

The demands for a gentle and effective agitation system for animal cells, fulfilling all requirements, are:

- maintaining of gentle mixing at supra-critical micro eddy diameter,

- short mixing times to guarantee homogeneity,
- sufficient surface renewal to ensure oxygen transfer ('back mixing') in the case of surface aeration
- compatibility of the agitation system to conventional stirred-tank bioreactors (flexibility).

Preliminary experiments with human melanoma cells in suspension culture were carried out in 2 litres stirred-tank bioreactors (Leist, 1988). Two six-bladed Rushton type impellers were placed on the stirrer shaft. Corresponding to Nos. 1 and 2 of the problem list, a clear decay of viability and μ was registered at stirrer speeds high enough to prevent cell sedimentation. No permissive window for the stirrer speed could be determined.

Therefore and with respect to the list of specifications stirred-tank bioreactors were converted into short loop bioreactors with draft tube and two four-bladed marine type impellers (Fig. 9). This kind of agitation solves the problems No. 1 to 3 of the problem list for the cultivation of human melanoma cells (Leist et al., 1986; Leist, 1988) and CHO cells (Kurano et al., 1990a, b).

- The application of marine type impellers for the agitation of Newtonian fluids could ideally result in laminar agitation (problem list No. 4.). An adequate mixing can be achieved by choosing a ratio of inner (Fig. 9A) and outer volume of the draft tube (Fig. 9B) slightly different from No. 1. The resulting different flow rates in the two compartments A and B lead to appropriate mixing of the suspension as proved by the presented experimental results (e.g., Fig. 8).
- The height of the overflow over the draft tube (height difference between medium level and the upper brim of the draft tube) determines effectively the oxygen uptake of the medium in the case of surface aeration. Decreasing that height difference reduces the exchange rate of the liquid surface. Therefore the oxygen uptake rate by back mixing of air is adequate to reach 2×10^7 cells per ml with human melanoma cells in a 5-litre bioreactor (Leist, 1988). A further increase in oxygen uptake could be achieved by supplying pure oxygen. This agitation and aeration system bypasses also the sensitivity of melanoma cells against gas bubbles (Leist, 1988).

Thus the remaining shear stress effects observed in the described culture system are mainly caused by micro eddies (problem list, 4.). A reduction of these effects can be achieved by adding 1 g l^{-1} CMC (carboxy methyl cellulose) to serum- and protein-free medium (Leist, 1988). Cultivation experiments in serum-free medium without CMC addition effected in either no growth or cell sedimentation, depending on the stirrer speed. However, no permissive window for the stirrer speed could be determined for STR-systems.

Two effects can be distinguished when comparing the results of experiments to determine the cell destruction by shear forces (Fig. 10) with the effects observed during long period cultures;

- Damage of the cells occurs within minutes if the stirrer speed exceeds the critical value (900 rpm) as described in Fig. 10 ('immediate cell damage'). Response consists of the reduction of cell number, viability and the appearance of cell debris in the culture fluid.

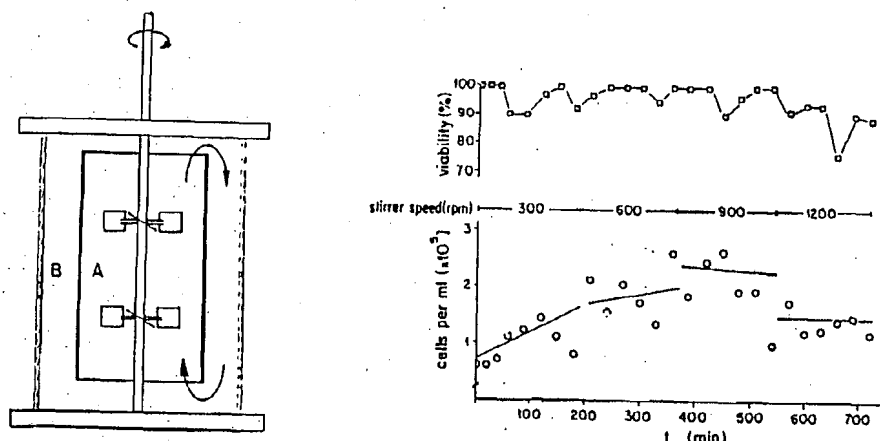


Fig. 9. Simplified section of a compact loop bioreactor for cultivation of animal cells. Shown are the agitation system existing of a draft tube and two four-bladed marine impellers. This system provides homogeneity and guarantees gentle revolution at defined degree of turbulence (depending on the ratio of inner and outer volume of the draft tube). At a maximal STS of 3 m s^{-1} for several hours, the shear tolerance of human melanoma cells was rather high (Leist, 1988) compared with other cell lines as CHO (Kurano et al., 1990a) or hybridomas (Schürch et al., 1988). With regard to the maximal tolerated stirrer speed, no differences between the three human melanoma cell lines B4, 84-12-26, and B4M16d have been observed (Leist, 1988). A marks the volume inside of the draft tube and B the reactor volume outside of the draft tube.

Fig. 10. Stirrer-speed-dependent damage of B4 cells in a 2 litre compact loop bioreactor (stirrer diameter: 48 mm). As a relation of time (t) and the stirrer speed are shown: cell concentration and viability (see also Leist et al., 1986; Leist, 1988). Four different stirrer speeds were managed each during 200 min. The decrease in cell number at a stirrer speed of less than 600 rpm results in beginning inhomogeneity. The decrease of cell viability and cell concentration at more than 900 rpm is clearly demonstrated.

- A 'long-term effect' is observed after weeks of acting shear forces and is manifested by an increase of q_s of substrate and a decrease of μ . This long-term effect is also reported by Harbour et al. (1988) and interpreted as 'shear stress'. The critical value for shear stress is much lower (see below).

Table 8 shows critical STS (stirrer-tip speed) for immediate cell damage and for shear stress of human melanoma (B4) cells in media with and without serum supplementation.

TABLE 8

Critical stirrer tip speed (STS) for immediate cell damage and for shear stress of B4 cells in serum free and serum containing (5% v/v FCS) medium

Effect	Critical STS RPMI serum-free	Critical STS RPMI + 5% FCS
Immediate cell damage	$> 3.5 \text{ m s}^{-1}$	$> 4.5 \text{ m s}^{-1}$
Shear stress	$> 1.0 \text{ m s}^{-1}$	$> 1.5 \text{ m s}^{-1}$

The critical values for shear stress effects are significantly lower than for immediate cell destruction. Long-term effects become manifest in very subtle and slow drifts of metabolic rates, including activation-deactivation of genes (genetic drift). However, shear forces are difficult to determine, simulate and predict in a given culture system. Some efforts have been undertaken to develop mathematical models for cellular reactions on shear forces (Schürch et al., 1988). Nevertheless they are still not fully understood, are multiple and need further investigations (Oh et al., 1989; Schürch et al., 1988). Diamond et al. (1989) describe a stimulation of t-PA production by human endothelial cells. Again this example points to very complex effects of the shear forces on metabolic control and the 'secondary' metabolism. Thus identification of shear force effects up to now has limited biological basis and modelling appears premature.

Table 8 shows a protective effect of serum. The most important finding is the fact, that the critical STSs in serum containing medium are up to 50% higher than without serum supplementation.

Cell damage by virtue of aeration occurs in suspension culture of all different human melanoma cell lines. In airlift systems cells were moved to surface and destroyed by exploding gas bubbles. The dead and the few remaining living cells formed a sticky ring at the glass wall of the bioreactor just above the liquid surface. A culture with B4 cells was treated with sparged air at a cell density of $1 \times 10^6 \text{ ml}^{-1}$ in exponential growth phase. The cell density was reduced overnight by a factor 10. The whole rest of the cells was sticking at the bioreactor wall, just on top of the liquid level. A rather high percentage ($\sim 30\%$) of these cells was still living. This leads to the assumption that the destruction of B4 cells is not caused by the gas bubbles directly but by cells sticking at the glass wall at the liquid surface. Therefore airlift systems often applied for animal cell culture (Bugarski et al., 1989; Carvell and Chapman, 1989) are not suitable for B4 cell culture. These facts show distinctly that generalizations in view of shear stress effects are inadequate.

Scale-up

Besides biological and technical feasibility, process scale-up depends on several economical factors, such as specific price of the product, worldwide demand and the number of competing producers. Titles of overview articles such as "Scale-up: how big is big enough?" (Van Brunt, 1988) or "Mammalian cell culture scale-up: is bigger better?" (Horvath, 1989) illustrate that the problem of scale-up (because of its numerous aspects) can be neither comprehensively discussed nor easily solved.

10000 and more liters working volume represent the production scale for a lot of bulk productions produced by microorganisms. These products are also called 'low value products'. Mammalian cell culture products consist however, of 'high value biologicals' with specific selling prices of up to 10^6 \$ per g. Examples are given in Table 1.

Although the figure of Van Brunt (1988) is based on data from 1984, it demonstrates not only the clear relation between selling prices and concentration in starting material, but also the very high specific prices of cell culture products, up to

10^9 \$ per kg. The figure shows also the potential of increasing specific productivity (resulting in an increased concentration in starting material) to reduce efficiently the selling prices. The selling prices for monoclonal antibodies, however (at least for therapeutically active antibodies), are with 10^5 to 10^6 \$ per kg underestimated by a factor 100 to 1000.

The worldwide market for a high-value product is likely to be several hundreds of kilograms at most. For instance Xoma Corporation's (Berkley, CA) product with the largest production needs is an antibody that treats septic shocks by binding and neutralizing the bacterial poisons that are released into bloodstream (Van Brunt, 1988). This antibody's worldwide market does not exceed 100 kg a year. Superoxide dismutase, a product of Chiron (Emeryville, CA), may have a market of hundreds of kilograms but not more (Van Brunt, 1988). A lot of other products, such as anti-clotting factors, lymphokines, growth factors, or vaccines are needed in much lower quantities.

Biological and technical reasons limit the scale of mammalian cell culture processes. Insufficient nutrient and oxygen supply, too high shear forces when trying to achieve complete mixing and/or inhomogeneity concerning all culture conditions, variable hydrostatic pressures in big bioreactors, inhomogeneity and many other factors arise to severe problems with increasing scale. Up to several thousands of liters these difficulties can be solved by the application of suspension culture technology. Therefore the biggest scales of up to 8000 litres have been realized by stirred-tank bioreactors (Pullen et al., 1985; Handa-Corrigan, 1989).

According to Diane Blumenthal (CalBio, Mountain View, CA), data generated in a 35 litres bioreactor are comfortably scalable to 2000 litres reactors (Van Brunt, 1988). Bliem and Katinger (1988) described the methodological schedule, models, and correlations of process variables of production process scale-up, up to 8000 litres.

The potential of 'optimization strategies' concerning bioreactor design, culture conditions, process control, specific productivity and/or cell density to reduce scale at constant or even increased product yield is usually underestimated. An exclusive increase of the specific productivity of a producer cell line reduces the production scale by a factor 10 or more, keeping product yield constant. Together with the low product demands and the high specific value, the production scale for most of the mammalian cell culture products is determined to range from 10 to several hundreds of litres (Van Brunt, 1988). This scale, however, is not already big enough for typical 'large scale problems' and has been applied very successfully in the last decade for the production of biologicals with mammalian cells (Reuveny et al., 1980; Bodo, 1981; Klein and Ricketts, 1981; Van Wezel et al., 1984; Morandi et al., 1985; Pay et al., 1985).

Validation of suspension culture technology

For human melanoma cells, suspension culture technology proved to be able to solve all primary and secondary problems mentioned above. Therefore the time has

TABLE 9
Classification of adherent and suspended culture methods divided into six different principles

Growth	Adherent				Suspended	
	Principle: surface stat. medium stat.	surface mov. medium stat.	surface stat. medium mov.	cells stat. medium mov.	surface mov. medium stat.	cells moved medium mov.
	Examples: T-flask multi tray plates tubes	roller bottle rotating disk gyrogen	perfus. syst.: hollow fiber solid bed ceramic unit	microcarrier liquid bed encapsulation cross linking	cell fixation: hollow fiber ceramic unit	suspension culture in different agitated bioreactors
<i>Analytical accessibility (on-line) of:</i>						
Biological	no	no	no	complicated	no	easy
Biochemical	complicated	complicated	no accuracy	easy	no accuracy	easy
Physical parameters	complicated	complicated	no accuracy	easy	no accuracy	easy
<i>Control of (in continuous processes):</i>						
μ	no	no	no	possibly	possibly	yes
μ indep. of D	no	no	no	no	no	yes
Cell number	no	no	no	possibly	no	yes
<i>Mode of operation:</i>						
Homogen.	no	no	no	approx.	no	yes
Continuous	no	no	yes	yes	yes	yes

Cell recycl.	per se	per se	per se	per se	per se	yes
Serum-free medium during complete process	no	no	no	no	possibly	yes
Max. cell density in batch culture (cells per ml)	10 ⁶	10 ⁶	-	2 × 10 ⁶	-	2 × 10 ⁶
Culture system:						
Construction	simple	simple	complex	complex	complex	simple
Labor effort for:						
building up	little	little	interm.-big	interm.-big	interm.-big	sim.-interm.
mainten.	big	big	big	big	big	little
Financial effort for:						
building up	little-interm.	little-interm.	interm.-big	interm.-big	interm.-big	little-interm.
mainten.	big	big	big	interm.-big	big	little-interm.
Biomass yield of medium	small	small	interm.-big	interm.-big	interm.-big	small-big
Degree of operational flexibility	low	low	low	interm.-high	low	high
Scale up as:						
Multi unit	yes	yes	yes	yes	yes	yes
Single unit	no	no	no	yes	no	yes
Max. working volume per unit (l)	0.2-2	0.5-2	0.2-50	500	0.2-50	8000
Effort	big	big	big	interm.	big	little-interm.
Suitable for:						
Process and medium developm.	no	no	no	no	no	yes
Application at production scale	no	no	no	yes	possibly	yes

Comparison and valuation correspond to different culture technical parameters and properties (Heath and Belfort, 1987; Butler, 1987; Spier, 1987; Kasting and Scheirer, 1985; Leist and Meussdoerffer, 1986; see also Leist, 1988).

come to validate suspension culture technology compared with adherent culture techniques. Also considerations of the great diversity of culture technologies have to be shown with respect to their application in research, development and production. To achieve a significant validation of suspension culture technique in the context of all adherent technologies, six different suspended and adherent technologies are listed in Table 9 and some important process parameters and relevant properties are compared.

Missing or non-feasible data acquisition characterizes adherent and immobilized suspended culture systems. Together with the formation of gradients, these properties markedly hinder adequate process control. Thereby the application of continuous process technique, requiring a sophisticated process control, becomes impossible. All advantages mentioned so far, including higher flexibility, arise suspension culture technology to the best suited system for process and medium development. Homogeneous mixing and the possibility of its application as a large-scale unit operation also makes a suspension culture become even more attractive for industrial application. Comparing maximal cell densities of batch cultures (without medium exchange) the frequently reported higher biomass yield in adherent systems will become irrelevant. Combining the chemostat technique with cell recycling (on serum- and protein-free defined medium!), the cell densities reached in adherent culture systems, even in serum-containing medium, could be matched (Leist, 1988). The question of serum addition becomes rather important for cost-yield-calculation of production and especially of downstream processing (isolation and cleaning). For human melanoma cells an integrated high-density production process based on a suspended chemostat technique combined with continuous cell recycling has been designed (Leist, 1988; Fig. 5) and successfully run for several weeks. Suspended culture systems could reach product yields that have recently been reported to be higher than those of immobilized systems (Ryll et al., 1990). Table 9 elucidates the exclusive potential of a suspension culture technique to solve all these problems.

Unfortunately, recent research and development in animal cell culture does not follow this conclusion. In particular the development of adherent culture systems shows a clear trend towards the most complex systems (e.g., hollow fiber and aeration systems; see also at the beginning of this article). The failure at the production scale of this technology is not surprising because of its high costs in maintenance, service and investment, and its loss of flexibility. Products such as interferons, interleukins, growth hormones, vaccines, and monoclonal and polyclonal antibodies produced in suspended systems elucidate the future importance of suspension culture technology at the production scale (Chapman et al., 1973; Mizrahi, 1981; Bodo, 1981; Klein and Ricketts, 1981; Familetti et al., 1981; Var Damme and Billiau, 1981; Rabin et al., 1981; Phillips et al., 1985; Witte et al., 1986; Backer et al., 1988; Handa-Corriggan, 1989).

For the realisation of the advantages, submerged culture technology has to be continuous. In industry, however, continuous processes are despised because of the problems of higher infection risk, genetic drift, cell line and product approval; nevertheless, they can be solved (Leist, 1988).

Genetic stability is an absolute prerequisite for high productivity, product qual-

ity, and product approval. This quality was achieved by homogeneous mixing and controlled culture conditions permitting only one genetic constitution to survive (Leist, 1988). Homogeneity can only be achieved in suspended systems, and only continuous operation of a culture system warrants constant culture conditions. Therefore the argument 'genetic stability' changes from the pretended reason for the application of batch processes to a cogent reason for continuous processes. A frequently reported solution (compromise) for this dilemma is the performance of repeated batch cultures, sometimes also called 'semi-continuous' cultures. This culture technique keeps at least the controlled culture parameters constant.

Costs for cleaning, maintenance and start up of the cultivation facility and operationally dead times are even reduced at continuous operation.

In view of these facts reconsideration of process strategies and objective definition appears necessary.

Conclusions and future prospects

For future hardware development progress has to be made in two directions, mainly in bioreactor technique and in process control. Several novel approaches are available for the cultivation of animal cells. Some of the recent developments may be of great interest (Lehmann et al., 1985; Bödeker, 1985; Leist, 1988). Low shear agitation systems as 'torus' bioreactors should be tested for the application for mammalian cell culture, also because of their high mass transfer rate at low energy uptake rate (Läderach and Widmer, 1984). A comparison of different bioreactors suitable for animal cell culture as done for prokaryotes and yeasts (Adler and Fiechter, 1983) would be important. These comparing characterizations would enable specific improvements at the investigated systems of interest. The design of more sophisticated culture technology would lead to 'high-performance bioreactors' as used for microbial cultivations (Sonnleitner and Fiechter, 1988). For process control, two factors are of basic interest: (1) on-line sensors for biological and biochemical parameters and (2) basic knowledge about 'cellular behaviour'. Despite great progress in the on-line determination of enzymes (Winqvist et al., 1988), physical and biochemical parameters (Merten and Palfi, 1987) and the successful application of biosensors for clinical analytics and bioprocess control. On-line detection, however, is still quite problematic, not only because of the analytics but also because of long-term stability and sterility of the sensors involved. Some progress has now been made in combining flow injection analysis with spectrophotometry or biosensors.

On the other hand, the fields of cell biology have shown great progress. As mentioned above, the design of biological test systems will be one of the big aims of the future. Therefore cellular metabolism and its regulation, as well as cell division and its regulation have to be determined in detail. The first efforts in this direction have been made (Kurano et al., 1990a, b; this issue). Kurano et al. describe the effects of physical, chemical and biochemical variables on the growth and metabolism of CHO cells. They have identified the formation mechanistics of lactate and

ammonia, two important cellular waste products, determined the kinetics, and developed a model for the control of these metabolic steps. The results, however, are a long way from giving a complete picture of the biological system 'animal cell', but they function as a trend setter and demonstrate the feasibility of such complex attempts.

Another field of progress not discussed in this article is the mass cultivation of plant cells. Plant cells show some advantageous properties with respect to their cultivation, such as a sensitivity to shear forces smaller than that of mammalian cells (Hooker et al., 1989). They do not request temperature control at 37°C and they are able to express and undertake post-translational modification or excrete proteins corresponding to patterns of higher eukaryotes. Their cultivation has recently been applied to the production of heterologous and/or animal and human genes. Interesting plant and mammalian proteins, their transforming vectors and synthesis with transgenic plant cells have been reviewed by Uchimiya et al. (1989). The interest in mass cultivation of plant cells was also stimulated by recent developments for plant regeneration to identical young plants (Shillito et al., 1989; Prioli and Söndahl, 1989) and the improvement of productivity by the generation of transgenic plants (Chupeau et al., 1989). Despite differences in metabolism, size and ultra structure and despite some advantages, the problems of cultivation of plant cells are similar to those of animal cell culture (Fiechter, 1980a, b, 1981; Fowler and Spier, 1984). Plant cells tend to aggregate irreversibly. This makes most of the measuring methods of biomass inapplicable. The media for plant cells consist of yeast extract, casein hydrolysate, coconut milk and plant hormones in addition to other complex additives. Suspension culture has been attempted in different types of bioreactors. Air lift, however, was most often applied. The first efforts to determine the effects of culture conditions such as shear forces have been realized (Hooker et al., 1989). Systematic process and medium development, however, has not been realized for plant cell culture.

Future animal cell culture technology will be applied in several fields of research, development, industrial production, as well as in human and veterinarian medical laboratories. The broad field of applications of cell culture products determines their high diversity in chemical nature. The trend is clearly towards high-value biologicals. The medical application of active biologicals such as fibrinolytics (e.g., t-PA) for site-directed thrombolysis (Tiefenbrunn and Sobel, 1989), growth factors for wound healing (Ten Dijke and Iwata, 1989), cancer therapy or human growth control, lymphokines, vaccines, antihemophilic factors and even oncogenes and their products for cancer diagnosis, prognosis and treatment (Huber, 1989) are as prominent as the administration of metabolism control enzymes (e.g., insulin). Al: immobilized enzymes and whole cells, acting as complex enzyme systems, will be introduced in medical therapy (Chang, 1988). Antibodies for medical diagnosis and therapy, such as tissue-specific carriers of therapeutically active compounds (Lawson et al., 1987) represent future products of an (at the moment) inestimable potential. Monoclonal antibodies recognizing cancer cell-specific surface molecules represent a further approach for future cancer recognition and treatment (Trauth et al., 1989). Moreover, antibodies will be applied in industrial fine-chemical purification, for instance in affinity chromatography or for analytical purposes.

The determination of residual DNA in biologicals produced by animal cell culture or recombinant technologies has become very important for product approvals. The most sensitive methods are based on hybridization of DNA with sequence-specific monoclonal antibodies (McKnabb et al., 1989), the latter being also a cell culture product.

The utilization of the cells themselves represents another approach. Special monolayer and multilayer epidermal cell culture technique delivers biotechnologically reconstituted human skin for skin surgery and for its utilization as in vitro test system (e.g., for cosmetics).

Another application of total cells in the near future is the in vitro propagation of lymphocytes (e.g., T-cells) in patients suffering from immune deficiency and the subsequent restoration of an increased amount of active lymphocytes to the blood circle of the same patients. This method will represent a potent weapon in the fight against AIDS and other diseases with reduced immune activity, and more important, without giving rise to antigenic problems.

The greatest future potential, though requiring the most detailed and sophisticated knowledge in cell biology, genetics, and biochemistry, bioreactor design and process control, is given by the employment of cell, tissue and organ culture techniques used as in vitro bio-test systems.

An indirectly related approach is the producing technology with transgenic animals and plants (Westphal, 1989; Chupeau et al., 1989; Uchimiya et al., 1989). The animals (mice, cows and sheep) or plants serve as 'bio'-reactors producing at present several transgenic products that are secreted in their milk or their seed, respectively (Gordon et al., 1987; Fox et al., 1989; Clark et al., 1989; Vandekerckhove et al., 1989; Behringer et al., 1989; Uchimiya et al., 1989). However, downstream processing of the very sensitive biologically active proteins from the complex mixture 'milk' is still problematic and a high product purity (free of viruses or mycoplasmas) of reproducible quality cannot be achieved. This means that product approval becomes more than questionable. Also, problems with sterility result in identical difficulties, and animals cost much more in acquisition and maintenance than bioreactors. Finally, involvement of animals in industrial applications becomes more and more unattractive for ethical and political reasons.

This article demonstrates obviously that cell culture cannot be substituted by recombinant microorganism culture or transgenic animals; but rather represents a complementation of these concepts. Within the animal, cell culture suspended culture systems have a great number of advantages over immobilized systems for large-scale applications.

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The growth of Vero cells in suspension as cell-aggregates in serum-free media

Jack Litwin

State Bacteriology Laboratory, 105 21 Stockholm, Sweden

Received 14 July 1992; accepted in revised form 8 October 1992

Key words: cell-aggregates, serum-free medium, suspension culture, Vero cells

Abstract

Vero cell lines, usually considered anchorage-dependent, could be grown as cell-aggregates in suspension culture with serum-free media. Several different combinations of base media gave growth results above 10^6 cells/ml (NCTC 135:SFRE 199-1; NCTC 135:Waymouth MB 752/1; NCTC 135:RPMI 1640). Insulin was not essential for growth and Bovine Serum Albumin could be diluted out of the media if linoleic acid was present. The size and density of the aggregates formed varied depending on the media used.

Introduction

The growth of animal cells in suspension as cell-aggregates holds many advantages for the scaling-up of these cells for the production of vaccines and biologicals (Tolbert, *et al.*, 1980; Litwin, 1991; Litwin, 1992). The possibility that anchorage-dependent cells can be grown in this form was shown by Litwin (1992) when a Vero cell line was grown as cell-aggregates in suspension culture with a serum-free medium containing alpha MEM:F12 (1:1) without the aid of micro-carriers (White & Ades, 1990), microspheres (Goetghebeur and Hu, 1991) or encapsulation techniques (Trépanier, *et al.*, 1990). However, another Vero cell line obtained from the WHO and standardized for human viral vaccine production did not grow well in this medium. This report describes the response of these cells in suspension culture with other serum-free media.

Materials and methods

Cell line

The Vero cells used in these experiments were obtained from the WHO and used for Polio virus vaccine production at the State Bacteriology Lab., Stockholm, Sweden. The author obtained the cells at passage 138. The cells were originally grown in MEM + 25 mM HEPES + 10% calf serum.

Serum-free media (SFM)

All base media were made up from powdered mixtures (all media and components were purchased from Sigma) and filtered sterilized through a 0.1μ membrane. The composition of the SFM is shown in Table 1.

Ferric choline citrate had been found in this

Table 1. The composition of serum-free medium used for Vero cell growth

	Base media (MEM, F-12, RPMI, etc.)
25 mM	HEPES
4 mM	Glutamine (final concentration)
1 mM	Na pyruvate (final concentration)
1 μ M	Ferric choline citrate
0.2%	Galactose (not used in medium containing 0.4% or more glucose. 0.1% glucose was added to Leibovitz L-15 medium, instead).
4 μ g/ml	Insulin
200 μ g/ml	Bovine Serum Albumin (BSA)
0.1%	Na bicarbonate
1:20 dilution	Serum Replacement Concentrate (SRC 3000)
	(Tissue Culture Services, Botolph Claydon, Buckingham, U.K.)
	Final pH 7.0-7.1

laboratory to be an excellent iron compound for replacing human transferrin for a number of cell lines. For Vero cells a concentration of 1 to 5 μ M gave similar growth results.

The following antibiotics were used: 100 μ g/ml benzyl penicillin, 100 μ g/ml Streptomycin sulphate, 10 μ g/ml Neomycin, 10 μ g/ml Gentamicin and 10 μ g/ml Tylosin tartrate. This mixture had no effect on cell growth.

The cells were adapted to SFM in stationary cultures by adding them directly from medium with 10% serum to SFM. The best SFM for anchorage-dependent Vero growth contained SFRE 199-1 or Leibovitz L-15 as base medium. These media alone or in combination were not suitable for the growth of Vero cells in suspension. The cells used to start spinner cultures had been passed at least 5 times in SFM.

MEM vitamins were added to Lactalbumin Hydrolysate (LAH) base medium. For the addition of only LAH to various media a 10% solution was made in 2N HCl as stock solution. After addition, the medium was neutralized with 10 N NaOH.

All mixtures of base media given in the text were 1:1.

Suspending cells from monolayer cultures

Cells in monolayer cultures were suspended with 20 μ g/ml Protease from *Streptomyces griseus* (Sigma) + 5 mM trisodium citrate in phosphate buffered saline without Ca^{2+} or Mg^{2+} .

Suspension cultures

Suspension cultures were started with either 50 ml or 100 ml SFM in a 1 liter spinner bottle. Half the medium was changed every 3-4 days by centrifuging the cells and adding them back with the fresh medium. Eventually, the volume of the medium was increased to 200 ml. Thereafter, half of the medium was changed three times a week by allowing the culture to remain stationary for 15-30 min, pipetting off the top half of the medium with a 100 ml pipette and then adding an equal volume of fresh medium. Very few cells were lost during this procedure because most of the cells grew in dense aggregates which settled out quickly.

Cell counts

A 2-ml aliquot was removed from each culture about twice a week. The cells were centrifuged and resuspended in an equal volume, or a multiple thereof, of a solution containing 42 g/l citric acid + 0.5 g/l crystal violet in distilled water. After 30 min with intermittent shaking the cell nuclei were counted in a Bürker chamber. Nuclei from presumably living cells retained some structure whereas nuclei from presumably dead cells were smaller and homogeneously stained. The nuclei from presumably dead cells were not counted. All counts were made by the same person.

Results

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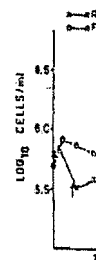


Fig. 1. The containing volume of n lin began to medium. Tr not includ

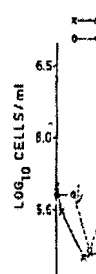


Fig. 2. The containing arrows.

Results

A test of 16 different commercially available base media showed that no one medium, by itself, would support growth above $1-3 \times 10^5$ cells/ml of the WHO Vero cells in suspension. However, higher values were obtained by combining various media (Figs. 1-4).

In all experiments there were indications that one could scale-up by doubling the volume of the medium (single stemmed arrows in figures). The cells also showed continued growth after insulin was gradually diluted out (double stemmed arrows in figures). When BSA was diluted out in the medium (triple stemmed arrows) the cells

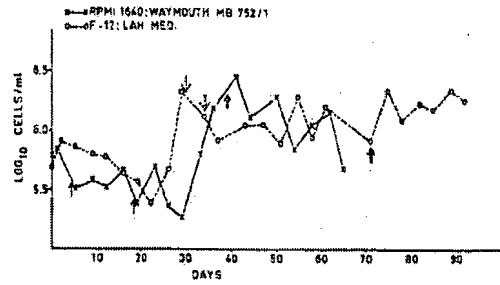


Fig. 1. The growth of Vero cells in suspension culture with SFM containing the base media indicated. Single stemmed arrow: volume of medium was doubled. Double stemmed arrow: insulin began to be diluted out by not including it when changing medium. Triple stemmed arrow: BSA began to be diluted out by not including it when changing medium.

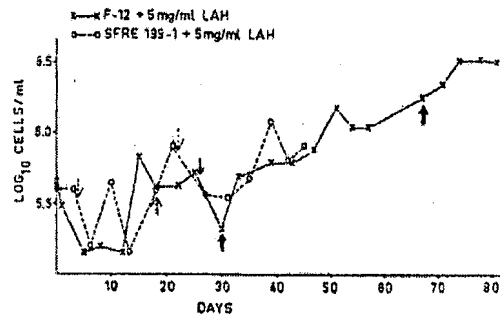


Fig. 2. The growth of Vero cells in suspension culture with SFM containing the base media indicated. See Fig. 1 for definition of arrows.

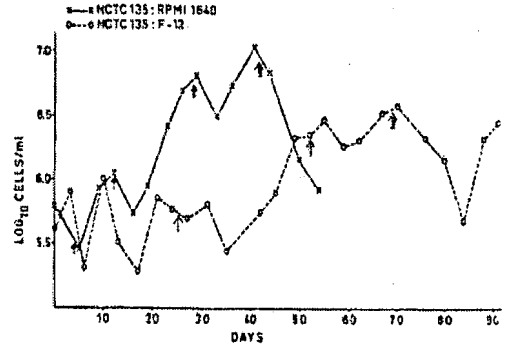


Fig. 3. The growth of Vero cells in suspension culture with SFM containing the base media indicated. See Fig. 1 for definition of arrows.

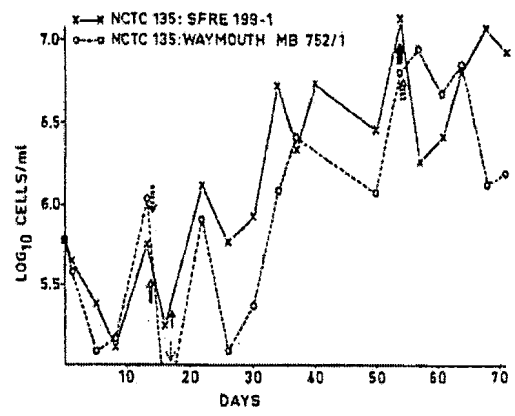


Fig. 4. The growth of Vero cells in suspension culture with SFM containing the base media indicated. See Fig. 1 for definition of arrows. When BSA began to be diluted out of the medium $1 \mu\text{g/ml}$ and $0.1 \mu\text{g/ml}$ linoleic acid was added to the cultures containing NCTC 135:Waymouth MB 752/1 and NCTC 135:SFRE 199-1, respectively.

could continue to survive and grow if linoleic acid was present in the medium as in F-12 (Figs. 1 and 3) or if this fatty acid was added to the media at a concentration of 0.1 to $1 \mu\text{g/ml}$ (Fig 4). Without the addition of linoleic acid increased number of cells died as BSA was diluted out in the medium (Fig. 3; Litwin, 1992).

The large variation in cell counts obtained with NCTC 135:SFRE 199-1 and NCTC 135:Way-

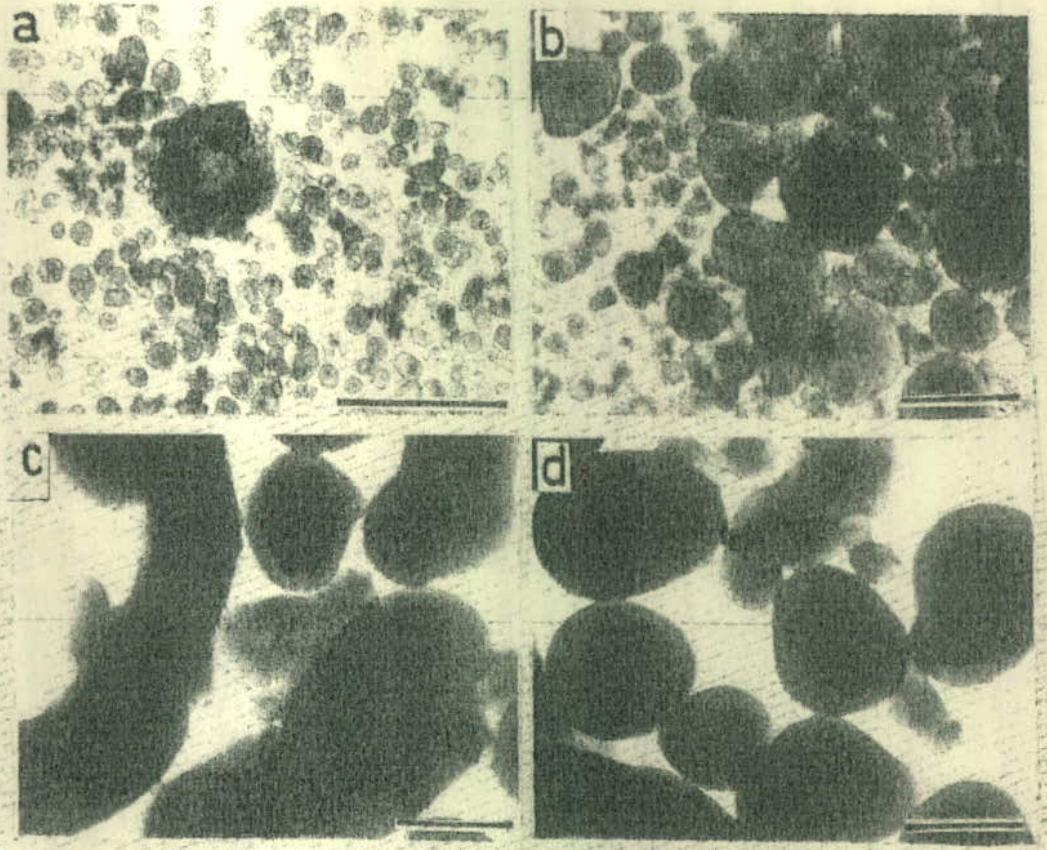


Fig. 6. (a-d) Vero cell-aggregates formed in suspension culture with SFM. (a) 52 days incubation in F-12 + 5 mg/ml LAH. (b) 63 days incubation in RPMI 1640:Waymouth MB 752/1. (c) 35 days incubation in NCTC 135:Waymouth MB 752/1. (d) 35 days incubation in NCTC 135:SFRE 199-1. The bar in each figure represents 1 mm.

There is a disadvantage if the aggregates become too large and dense resulting in necrosis and cell death in the center. To avoid necrosis and to obtain the best growth throughout the aggregate, it has been estimated that they should be no larger than about 300 μm in diameter (Folkman and Greenspan, 1975; Durand, 1990; Schwachöfer, 1990). To utilize this technique most effectively a greater knowledge must be obtained about the mechanisms and substances which control or influence aggregate formation, size and cell density. Our observations suggests that these variables are dependent in some way on components in

the medium.

Tolbert *et al.* (1980) had shown that cell-aggregate suspension cultures could be used for the production of biologicals. The close proximity of cells in aggregates should also allow for the efficient spread of virus for vaccine production, which is suggested by the results of Trépanier *et al.* (1990).

The results from the present experiments further substantiate that suspension cultures of cell-aggregates may be an important alternative method for large-scale cell cultivation for the production of vaccines and other biologicals.

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Key words
tocopherol

Abstract

Transformant
medium
supplement
Cell death
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involving
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Introduction

The BHK-21
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