On-line Monitoring and Control of Animal-Cell Cultures

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op vrijdag 1 november 1996
des namiddags te vier uur in de aula

Stellingen

1. Wanneer een meetapparaat de concentratie van een substantie goed weergeeft in een bufferoplossing, kan nog geen uitspraak gedaan worden of dit apparaat geschikt is voor online procesanalyse.

   Schürgerl, K. (1993) Which requirements do flow injection analyzer/biosensor systems have to meet for controlling the bioprocess? J. Biotechnol. 31, 241-256.

2. 12 metingen in 11 dagen, respectivelijk 23 metingen in 22 dagen, zijn nog geen bewijs dat de gebruikte analysemethoden stabiel genoeg zijn voor de kontrole van een dierlijke celkweek.

   Renneberg; R. et al. (1991) Enzyme sensor-FIA-system for on-line monitoring of glucose, lactate and glutamine in animal cell cultures. J. Biotechnol. 21, 173-186.

3. Het feit dat een EU-burger die in een ander EU-land woont, verplicht wordt tot het inwisselen van zijn EU-rijbewijs voor een ander EU-rijbewijs zegt meer over de eenheid in de EU dan alle officiële verdragen.

4. Een persoon die een taal niet volledig beheerst, is niet automatisch dom.

5. Fermentoren voor vaste-stoffermentaties zijn technisch eenvoudige apparaten, omdat vele factoren die van belang zijn voor de productie niet bekend zijn.

6. Ondernemingen met tienduizenden werknemers roepen automatisch een profilerings drang bij individuele werknemers op.

Stellingen behorende bij het proefschrift "On-line Monitoring and Control of Animal-Cell Cultures".

J.J. van der Pol
Wageningen, 01 November 1996

aan mijn ouders

Vorwort

Diese Arbeit konnte nur mit Unterstützung und hohem Einsatz von vielen Personen entstehen. Als erstes möchte ich Herrn Wandrey und Hans Tramper für ihr Engagement und ihre Bereitschaft, diese Deutsch-Niederländische Promotion (und Experiment) zu einem guten Abschluß zu bringen, danken. Zwei weitere Personen, die mir beim Schreiben der Veröffentlichungen viel Hilfe geleistet haben, waren Manfred Biselli und Kees de Gooijer. Manfred vielen Dank, Kees 'bedankt'. Furthermore, I am deeply grateful to Simon Livings and Allison Reid who made this thesis legible.

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wünsche ihm viel Erfolg bei seiner Promotion. Ich danke außerdem "meinen" ehemaligen HIWI's Anja Schmitz und Michael Slama, die für FIA ebenfalls großen Enthusiasmus zeigten. Auch ihnen wünsche ich viel Erfolg bei ihrer Doktorarbeit. Cornelia Herfurth führte alle Aminosäurenanalysen durch und sorgte immer für eine "nette" Stimmung. Helga Schmitz war immer wieder bereit, Vorkulturen anzusetzen und mich auch in anderen Dingen zu unterstützen. Christoph Born unterstützte mich bei den Modellierungen. Wenn die FIA-Kiste mal wieder "Voodoo"-Erscheinungen zeigte, waren Erik und Erich von der Elektronikwerkstatt immer hilfsbereit. Vielen Dank auch allen anderen Personen von der Elektronik- und Mechanikwerkstatt, welche für meine Experimente die verschiedensten Teile hergestellt haben.

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Contents

Chapter 1

Animal-Cell Cultures

INTRODUCTION

The expression "Animal-cell culture" describes the in vitro (in "test tube") culture of cells which are usually isolated from organs of mammals. For example, some established cell lines were isolated originally from the ovary of Chinese hamsters (CHO) and from the kidney of baby hamsters (BHK). The development of in vitro culture and of genetic techniques for the expression of foreign genes in animal cells (Sanders, 1990), gave access to higher quantities of glycosylated proteins (glycoproteins) for the first time. This development was followed by the discovery of new pharmaceuticals which could be produced by animal-cell culture on a large scale. In 1992, the sales of medically applied glycoproteins exceeded 2.8 billion US dollar world wide (Table 1), and the prospects are that this market will increase rapidly.

A second group of animal cells are hybridoma cells, which are formed by a fusion between lymphoid and myeloma cells (Köhler and Milstein, 1975). In these hybrid cells, the capacity for producing antibodies from the lymphocyte is combined with the immortality of the myeloma cell. Since a clone of one hybridoma cell produces identical antibodies, the invention of Köhler and Milstein allowed the production of Monoclonal AntiBodies (MAB) on a large scale.

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Antibodies are also glycoproteins and have a high affinity for one particular molecular structure. This feature makes monoclonal antibodies very suitable for the use in diagnostic tests. Other applications include as in vivo carriers for therapeutics (magic bullets) or for markers (imaging of tumors) and as the "catcher" for purification (affinity chromatography). The sales of MABs for application in diagnostic tests are expected to exceed 20 billion US dollars in the beginning of the next century (Baron, 1994).

Table 1. Estimated world wide sales of selected pharmaceuticals produced with animal cells in 1992 (Klausner, 1993).

Product	Sales million US $
Alpha-interferon	565
Beta-interferon	20
Gamma-interferon	25
Erythropoietin	1225
Factor VIII	235
Human growth hormone	575
Interleukin 2	20
Tissue Plasminogen Activator (t-PA)	230

Due to the possibilities for genetic engineering, proteins can also be produced with micro-organisms. However, animal cells still have a number of advantages over production with genetically engineered micro-organisms, such as lower recovery costs (Datar *et al.*, 1993) and glycosylation patterns similar to that of human proteins (Morandi and Valeri, 1988). The glycosylation pattern has a great impact on the physicochemical properties, the activity and stability of the protein in vitro (Schachter, 1986), and it determines the efficiency of the pharmaceutical in vivo (Jenkins and Curling, 1994).

THE CULTIVATION OF ANIMAL CELLS

The environment of mammalian cells in the living organism is strictly controlled between narrow limits. Moreover, specialization of the cells (differentiation) make them dependent on compounds produced by cells from other parts of the organism. Consequently, animal cells cultivated outside the organism can only survive if supplied with a similar environment, in other words a complex culture medium. Physicochemical parameters such as osmolarity, temperature and pH can only be varied between narrow limits (Werner *et al.*, 1992). The culture media used in animal-cell cultures are therefore complex and expensive cocktails of salts, amino acids, glucose, vitamins, trace elements, growth hormones and fatty acids, etc. Often serum is added, which contains unidentified compounds.

Characteristic of animal cells are low growth and production rates in comparison to micro-organisms, and their sensitivity to shear forces since they lack a cell wall. Anchorage-dependent cell lines need also a surface to grow. This slow growth in combination with the rich media forces the use of strict sterile conditions during the culture of animal cells.

Culture methods for the production of larger quantities of glycoproteins can be divided into batch, fed-batch and perfusion culture (Fig. 1). *Batch* cultures are carried out without adding fresh medium. Consequently, the culture has to be terminated after several days, because growth and production stops after a substrate is depleted or an inhibitor exceeds its threshold concentration. Batch cultures of animal cells in stirred-tank and air-lift reactors have been commercially applied for the production of antibodies (Birch *et al.*, 1987; Backer *et al.*, 1988) and t-PA in reactor volumes up to 10 m^3 (Nelson, 1988). The advantages of batch cultures are its simplicity and the relatively low risk of contamination. The uncontrolled and transient media concentrations and the low volumetric productivity as a result of the short production period at a high cell concentration are the negative aspects of this culture method.

During a *fed-batch* culture, fresh medium is added to the culture until the maximum reactor volume is reached. The medium addition enables a better regulation of substrate concentrations than can be achieved by batch

cultures and it increases the product concentration by extending the maintenance of the viable cells. On a laboratory scale, the product concentration was enhanced up to 10 times when compared to batch cultures (Bushell *et al.*, 1994; Kurokawa *et al.*, 1994; Robinson *et al.*, 1994; Xie and Wang, 1994a and 1994b; Zhou *et al.*, 1995). However, controlled medium supply is necessary to reach the optimal conditions for production, which complicates the management of the culture.

Continuous *perfusion* cultures are characterized by the constant supply and removal of medium, whereby the cells are retained in the reactor by either separation, immurement or entrapment (Griffiths, 1992). This enables a production under steady-state conditions and with high cell densities for periods of several weeks or more. The medium concentrations can be adjusted to the optimal production conditions by changing the perfusion rate. Due to the high cell densities, high space/time yields can be obtained, which reduce the reactor volume required. The disadvantages are the relatively long start-up period and a high risk of contamination due to the complex hardware configuration and the medium supply. An additional problem can be the deterioration of the production due to genetic instability of the cell line (Martens *et al.*, 1993; Werner *et al.*, 1992).

The large number of different perfusion systems can be distinguished by the method applied to retain the cells in the reactor (Griffiths, 1992). *Separation* systems consist in general of a stirred-tank reactor with an internal or external separation device. The cell retention is achieved by separation of the cells from the spent medium. The separation is carried out using filtration (membranes or spin filters), sedimentation or centrifugation. Cell densities up to $3*10^7$ can be obtained with these systems (Hansen *et al.*, 1993; Kawahara *et al.*, 1994; Shi *et al.*, 1993; Tokashiki *et al.*, 1990). Commercial production in reactor volumes of up to 500 l with an external filtration unit have been described (Tolbert *et al.*, 1987; Bödeker *et al.*, 1994). A disadvantage of membranes and spin filters is that fouling can limit the culture period (Esclade *et al.*, 1991; Kawahara *et al.*, 1994). The scale-up of the other two separation systems is questionable, as sedimentation needs large volumes and centrifugation is very complex.

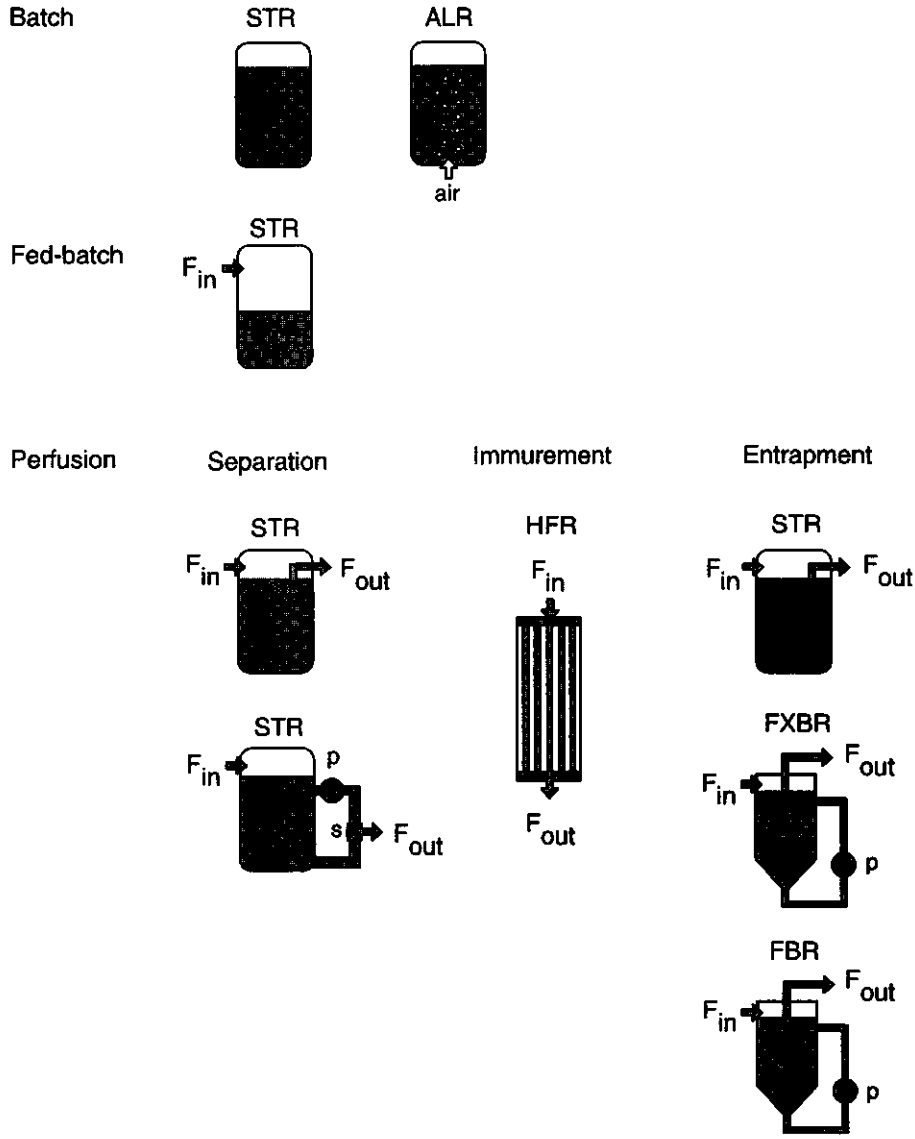

Fig. 1: Culture methods for animal cells (see text). F_{in} = medium feed, F_{out} = product effluent, p = pump, s = separation unit, STR = Stirred-Tank Reactor, ALR = Air-Lift Reactor, HFR = Hollow-Fiber Reactor, FXBR = FiXed-Bed Reactor, FBR = Fluidized-Bed Reactor.

Culture systems based on *immurement* are characterized by the complete enclosure of the cells. The most frequently used systems are hollow-fiber units. In these units, the cells are immured in the extra-capillary space, and the medium is supplied through the fibers. Cell densities of $1*10^8$ cells ml^{-1} and more have been reported (Hirschel and Gruenberg, 1987; Klerx *et al.*, 1988; Van Erp *et al.*, 1991). Hollow-fiber reactors can however only be applied for production on a small scale, since further scale-up is limited by gradients of medium compounds inside the cartridges (Chresand *et al.*, 1988; Nelson, 1988). These gradients are due to relatively low mass transfer rates at high cell densities (Hirschel and Gruenberg, 1987).

Entrapment is characterized by immobilization of the cells on a surface. A high surface to volume ratio and corresponding high cell densities are obtained with open-porous microcarriers. These have the additional advantage that the cells immobilized inside the microcarrier are protected against shear forces. Cells immobilized in macroporous microcarriers may be cultured in stirred-tank, fixed-bed or fluidized-bed reactors. With different cell lines, cell densities of approximately $1*10^8$ cells (ml settled carrier volume)$^{-1}$ have been obtained for a fluidized-bed reactor (Kratje and Wagner, 1992) and $2.0*10^7$ cells (ml settled carrier volume)$^{-1}$ for fixed-bed reactors (Racher and Griffiths, 1993; Griffiths and Racher, 1994). The mass transfer rate to the carrier is vital for cultures with microcarriers. When the transfer rate is too low, the cells deeper in the microcarrier will experience lower or in extreme cases zero substrate concentrations resulting in reduced metabolic activity or cell death. Microcarriers in stirred-tank (Tolbert *et al.*, 1987) and fluidized-bed reactors (Dean *et al.*, 1987) have been applied for the commercial production of glycoproteins.

THE METABOLISM OF ANIMAL CELLS

The very complex metabolism of animal cells is still not entirely elucidated. Moreover, the complex media with additional serum make a

complete identification of all metabolites affecting growth and production, practically impossible. The most significant metabolites for growth and production have however been classified. These key compounds can be divided into energy substrates (glucose, glutamine and oxygen); essential substrates (amino acids and vitamins); potential inhibitors (ammonium and lactate) and growth factors (Newland, 1990).

Glucose is used by animal cells for the generation of energy (ATP), precursors for nucleotides (ribose) and glycosylation sugars, and reducing equivalents for biosynthesis (NADPH). The major part of the consumed glucose is directly metabolized to pyruvate by the glycolysis (Sharfstein *et al.*, 1994) (Fig. 2). Pyruvate can either enter the TriCarboxylic-Acid (TCA) cycle resulting in its complete oxidation into H_2O and CO_2 or be converted to lactate by lactate dehydrogenase. The complete degradation to pyruvate yields 18 times more energy than its conversion to lactate. Ribose and NADPH are produced by the so-called pentose shunt.

Glutamine is an important energy (Reitzer *et al.*, 1979; Jenkins *et al.*, 1992) and nitrogen source for animal cells. Glutamine is first deaminated to glutamate by glutaminase and this results in the release of one molecule of ammonium (Fig. 2). In a successive reaction, glutamate is further converted to α-ketoglutarate. This conversion is catalyzed either by glutamate dehydrogenase releasing a second ammonium ion, or an aminotransferase forming either alanine or aspartate from pyruvate or oxaloacetate, respectively. In hybridoma cell lines, glutamate is preferentially converted by the transaminase pathway (Newland *et al.*, 1990; Jenkins *et al.*, 1992). In the TCA-cycle, glutamine can either be fully degraded to CO_2 and H_2O, or be converted to pyruvate by malate dehydrogenase and subsequently then converted to lactate (Sharfstein *et al.*, 1994; Zielke *et al.*, 1980).

Oxygen is the terminal electron acceptor of the oxidative phosphorylation process, by which the major part of the energy is generated (ATP) when glucose and glutamine are completely oxidized to H_2O and CO_2.

Amino acids are the building blocks of proteins and are therefore a prerequisite for growth and production of glycoproteins. Since animal cells are incapable of producing several amino acids, these essential amino acids

must be supplied. Amino acids can also be oxidized for energy by entering the TCA cycle (Fig. 3) (Hiller *et al.*, 1994), or function as precursor for the synthesis of other cell components.

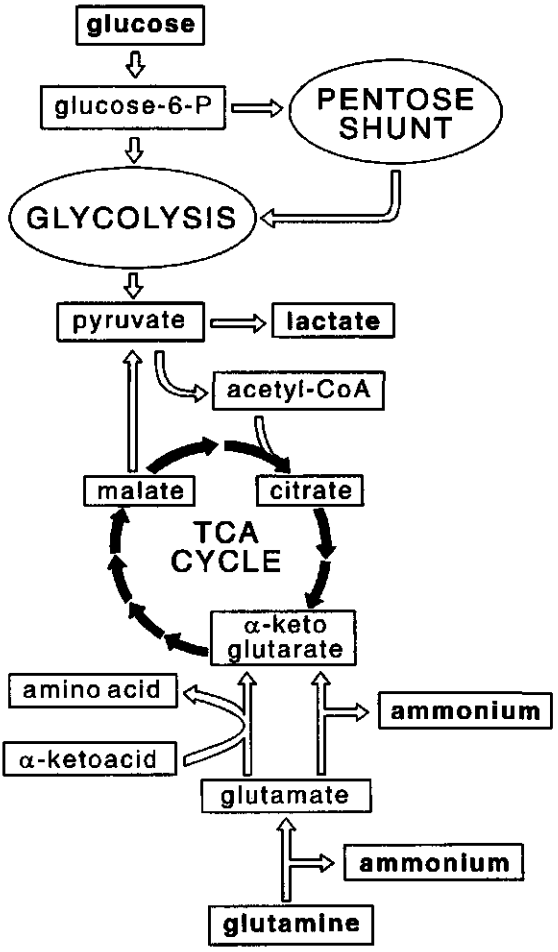

Fig. 2. Simplified metabolic map of the pathways for glucose and glutamine degradation. TCA = TriCarboxylic Acid, glucose-6-P = glucose-6-phosphate.

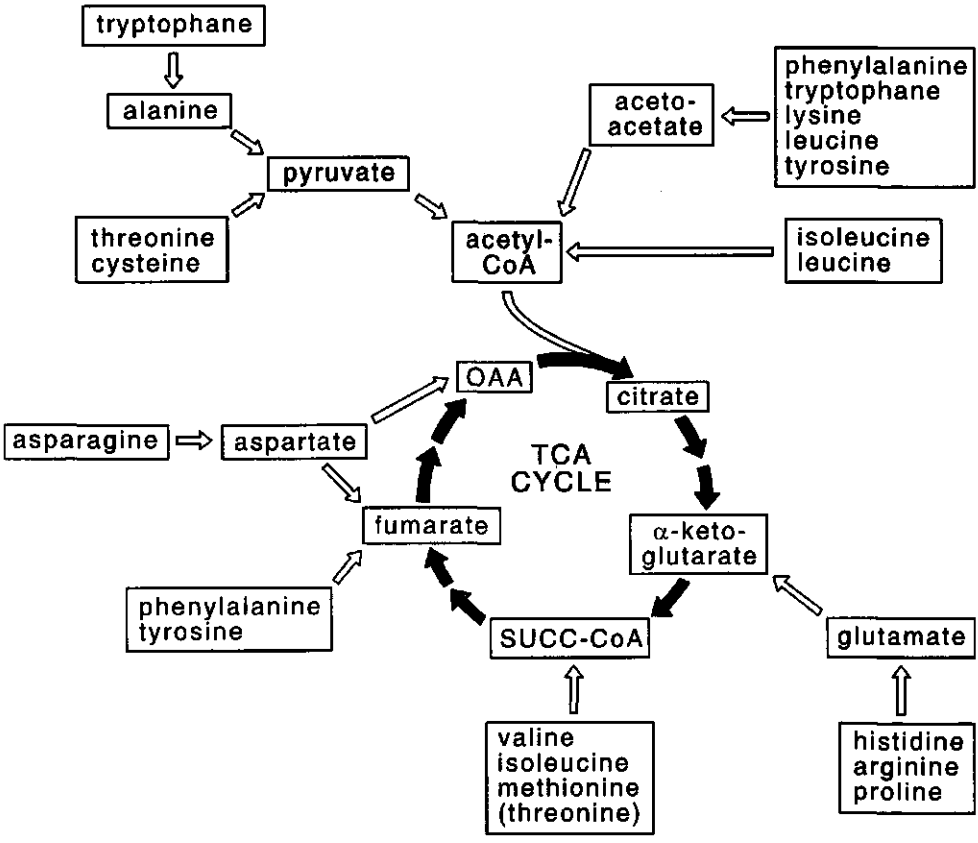

Fig. 3: A simplified metabolic map of pathways involved in the utilization of amino acids by the TriCarboxylic Acid (TCA) cycle and associated reactions according to Hiller *et al.* (1994). OAA = oxaloacetate, SUCC-CoA = Succinyl-CoA.

Ammonium is mainly the end product of the catabolism of glutamine (Fig. 2). Ammonium inhibits the growth of several cell types (Glacken *et al.*, 1986 and 1988), which results also in a lower productivity. However, ammonium has no influence on the specific productivity of hybridoma cells (McQueen and Bailey, 1990; Newland *et al.*, 1994; Ozturk *et al.*, 1992). The concentration at which ammonium decreases the growth rate by 50 %

varies between cell lines from 2 mM to 10 mM (Ozturk *et al.*, 1992). Ammonium also affected the glycosylation patterns of mammalian cells (Thorens and Vassalli, 1986; Borys *et al.*, 1994). Ammonium exerts its influence on the cell metabolism by increasing the intracellular pool of UDP-aminohexoses (Ryll *et al.*, 1994), which probably reduce the growth rate (Ryll and Wagner, 1992).

Lactate is the end product of the glucose and glutamine catabolism (Fig. 2). Lactate only inhibits the growth rate significantly at elevated concentrations, e.g. 55 mM for a hybridoma cell line (Ozturk *et al.*, 1992), which can be due to the higher osmolarity (Kurano *et al.*, 1990). Moreover, lactate inhibited the specific monoclonal-antibody production of a hybridoma cell line (Glacken *et al.*, 1988).

ON-LINE MONITORING AND CONTROL OF ANIMAL CELLS

Control of the environment in which cells are cultivated enables the optimal adjustment of conditions for growth and production. Moreover, process control is an important aspect of defining and characterizing the resulting product for license regulations (Liu, 1992).

In batch cultures only parameters such as dissolved-oxygen and carbon-dioxide concentration, pH and temperature can be controlled. Fed-batch and perfusion cultures offer the possibility to regulate substrate and/or inhibitor concentrations. This can be achieved in different ways (Fig. 4). The simplest method is to reduce the substrate concentrations in the medium feed (Ljunggren and Häggström, 1994), which results in relatively constant and low substrate concentrations in the reactor. Another method is to use a model of the cell metabolism either to predict the volumetric metabolic rate of the controlled component and consequently adapt the medium feed rate to the predicted metabolic rate (Xie and Wang, 1994a; Glacken *et al.*, 1989), or to relate the medium feed rate to the changing production or consumption rate of an easily measurable parameter, for example carbon dioxide (Tremblay *et al.*, 1993) or oxygen (Eyer *et al.*, 1995). The complex metabolism of animal cells however makes it difficult

to give an accurate prediction of these parameters and/or the relationship between them. The most reliable method is to analyze on-line the concentrations of the metabolites and adapt the medium feed rate to measured concentrations (closed-loop control). One disadvantage of on-line analysis is that an interface between the sterile reactor and the non-sterile surroundings, e.g. sample system, is required, since analysis inside the reactor is not possible. The risk of contamination through the continuous sampling and the lack of robust and reliable analytical systems mean that closed-loop control of substrate and inhibitor concentrations is seldom seen in industry. However, recent studies have shown the feasibility of closed-loop control of animal-cell cultures with HPLC (Kurokawa *et al.*, 1994) and flow-injection analysis (Van der Pol *et al.*, 1995a) for periods up to 900 h.

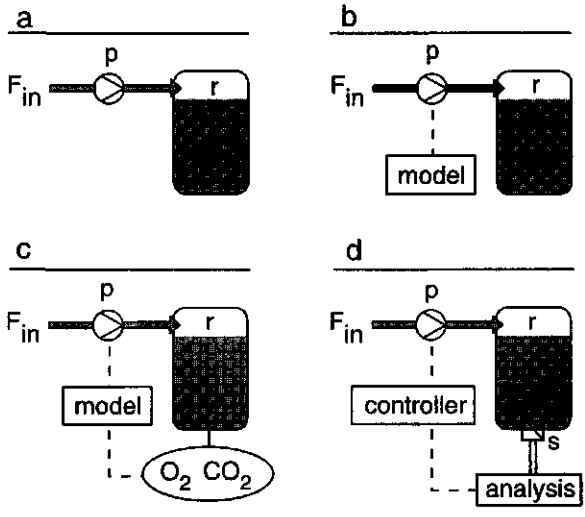

Fig. 4. Schematic representation of the possibilities for the control of substrate and/or inhibitor concentrations. a: Limitation of substrate concentration in medium feed (F_{in}). b: Adaptation of medium feed rate to changing volumetric metabolic rate predicted with a model. c: Relating the medium feed rate to O_2 consumption or CO_2 production rate by a model. d: Closed-loop control. p = medium pump, r = reactor, s = sample system.

On-line monitoring of the product quality, for example glycosylation patterns, and quantity enables to avoid ineffective production periods and facilitates process validation. Analysis of glycoproteins is normally carried out with immunoassays of which the best known is the Enzyme-Linked Immuno-Sorbent Assay (ELISA). Although several immunoassays have been automated to enable on-line monitoring of the product concentration (Gübitz and Shellum, 1993; Middendorf *et al.*, 1993), this is seldom applied in industry for the same reasons as those for closed-loop control. The attitude of industry could be changed if it is demonstrated that commercial analysis systems can be adapted for on-line monitoring as shown by Van der Pol *et al.* (1995b).

CONCLUSIONS

The relatively high production costs of glycoproteins using animal-cell cultures resulting from the complex media and low productivity, can be decreased by applying more efficient culture methods, such as fed-batch and perfusion. A further optimization of the efficiency can be achieved by on-line control, preferably closed-loop control, of the metabolite concentrations since these affect both the productivity and growth rate of animal cells. Moreover, on-line monitoring of the product concentration will be useful to minimize or avoid inefficient production periods and to validate the process. The integration of these aspects into the production process will make it competitive in the fast increasing market for glycoproteins.

OBJECTIVE OF THIS THESIS

In this thesis, analytical methods have been developed for the on-line monitoring and control of animal-cell cultures. The on-line measurement of metabolites is carried out with a newly developed flow-injection analysis device. Enzymatic assays for glucose, glutamine and lactate and a chemical

assay for ammonia are integrated into the analytical system. Furthermore, an analytical set-up with a newly designed sample system is developed for the on-line determination of antibodies. The long-term stability of both analytical devices is tested during cultures of immobilized hybridoma cells in a fluidized-bed reactor. Moreover, the multi-channel flow-injection analysis device is used for the control of the glucose and glutamine concentration in the same culture system.

OUTLINE OF THIS THESIS

In Chapter 2 of this thesis, the multi-channel flow-injection analysis system and its possibilities are described. The stability of the analytical device is tested in conjunction with long-term cultures of hybridoma cells in Chapter 3. During one of these cultures, the influence of the dissolved-oxygen concentration on the metabolism of the immobilized cells is investigated. In Chapter 4, the analytical device is integrated into a closed-loop control system to control the glucose and/or glutamine concentration. Both substrates are compared as control substrate for the medium feed rate during the start-up of hybridoma cultures. Furthermore, the influence of the perfusion rate on specific monoclonal antibody production during long-term culture is investigated. In Chapter 5, the monoclonal-antibody concentration is monitored on-line for more than 800 h. During the culture, the temperature is varied to analyze its influence on the metabolism of hybridoma cells. Chapter 6 gives a general overview of the possibilities of flow-injection analysis and underlines the factors which affect the stability of these analytical systems.

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Chapter 2

An Automated System for Multi-Channel Flow-Injection Analysis

SUMMARY

An automated multi-channel flow-injection analyzer for both new Flow-Injection Analysis (FIA) procedures and for process monitoring of animal and microbial cultures has been developed. The software package FIACCO (Flow-Injection Analysis Control and Configuration) was developed for hardware control, signal evaluation and long-term recording of on-line calculated results. The independent control of all active hardware components guaranteed great flexibility and allowed the implementation of a wide variety of FIA-methods. Flow-rate control was applied to govern the analyte conversion in packed-bed enzyme reactors. A significant increase of the peak width could be avoided in this way. A maximum of six enzyme reactors in the analytical system could be put in parallel sequence for the sequential determination of different analytes. Only one fluorescence detector was necessary for multi-component analysis as a result of selection of the enzymatic assays and/or chemical assays. Adjustment of the optimum pH for each assay was achieved by mixing two carrier solutions.

This newly developed analytical system was successfully applied to the on-line process monitoring of five key-components: glucose, glutamine, glutamate, ammonium and lactate in an animal-cell culture.

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INTRODUCTION

Despite of the wide acceptance of Flow-Injection Analysis (FIA) and its obvious advantages, the ability to determine more than two components with the same FIA set-up is still limited (Van der Linden, 1986). Especially solutions from food processing and culture media very often need multi-component analysis. To obtain a multi-component analyzer, single channels can be combined in one FIA set-up. Nielsen *et al.* (1990) and Nikalajsen *et al.* (1988) realized in this way a 4-channel FIA set-up. The relatively high costs per channel were justifiable in view of the successful on-line control of actual processes. Lazaro *et al.* (1986) proposed multi-wavelength detection with a diode-array UV-visible detector for multi-component analysis. Alternative methods are the arrangement of several detectors in serial or parallel configurations, and arrangements with different ion-sensitive electrodes (Bos *et al.*, 1990; Camman, 1988; Cardwell *et al.*, 1988).

For food and bioprocess analysis, enzymatic assays are of particular interest. Renneberg *et al.* (1991) applied an arrangement of different biosensors for the simultaneous determination of glucose, glutamine and lactate in animal-cell cultures. Hundeck *et al.* (1990) described an enzyme thermistor with four parallel enzyme-reactor cartridges in one FIA set-up. Ogbomo *et al.* (1991) and Kittsteiner-Eberle *et al.* (1989) designed a FIA manifold with parallel enzyme reactors using reagent injection. One disadvantage of these multi-channel systems is that the possibility to adjust the optimum reaction conditions for each enzymatic assay is restricted. The FIA set-up with reagent injection also has a relatively high consumption of sample solutions. Another major obstacle to the more general and flexible application of multi-channel FIA set-ups is the lack of suitable and reliable software and the insufficient reliability of the corresponding commercial hardware configurations presently available.

The aim of our work was to develop a flexible, fully automated FIA analyzer for enzymatic multi-component analysis. This flexibility should be extended to a wide range of automatically adjustable reaction conditions, especially reagent concentrations, pH values and residence times.

MATERIAL AND METHODS

Instrumentation and system concept

The general system concept of the multi-channel FIA system was developed on the basis of an earlier FIA set-up (Spohn *et al.*, 1991; Joksch *et al.*, 1991). The FIA system was fully automated and was thermostatically controlled at 15 °C (except for the FIA set-up shown in Fig. 10). The configuration of the hardware components was adapted to requirements of the particular analysis.

All hardware components (Table 1) were controlled by an IBM compatible Personal Computer (PC, processor 80386, coprocessor 80387). The PC was equipped with an A/D-converter card (PC-74, Meilhaus, Puchheim, Germany), a reed-relais board (PC-63, Meilhaus) and an eight fold RS232 serial interface card (PC-31, Meilhaus). All active components could also be controlled manually from the PC keyboard.

The A/D-converter card (12 bit) had eight differential signal inputs. The reed-relais board allowed the independent control of a maximum of 16 relais (24 VDC), setting six magnetic-switching, 3/2-way valves (Type 368/1/3/24/30, Akzo, Ratingen, Germany), two pneumatically actuated injection valves (Type 9010, Rheodyne, Cotrati, USA) and two 6-way valves (Type 9060, Rheodyne). Other relais outputs allowed the automatic selection of two pump rates of the peristaltic sampling pump (SP-GL 70, Meredos, Göttingen, Germany), preadjusted steplessly by two precision potentiometers and a time-controlled on/off switch. Another output served for the reed-relais-based switching of the detector autozero.

The eight fold RS 232 serial interface card was used to automatically control of four precision piston pumps P1-P4 (Dosimat 665, Metrohm, Herisau, Switzerland). The pumps were controlled independently of each other with respect to the flow rates ($\dot{V}_{1\text{-}4}$) and to the microprocessor commands GO, STOP, FILL and PUMP RATE. The commands STOP and GO allowed switching between four different carrier solutions synchronously, which resulted in a fast exchange of carrier solutions. Moreover, the command STOP made a very flexible implementation of single and multiple stopped-flow FIA procedures possible. The piston pumps were refilled after each determination to achieve maximum stability of the flow rates.

The pump rates of the piston pumps were software controlled during the determination procedure and these could be changed in steps of 2 µl min^{-1}. A maximum of eight different pump rates could be preadjusted and changed automatically during each time course and every 10 s with a time shift of 0.5 s. This enabled the adjustment of the mixing ratios of carrier-solution pairs between 0.1 - 10.

Table 1. Hardware components of the FIA-system.

Active hardware components	Number	Interface	Control variables and commands
Piston pump	4	RS 232 card (PC-31)	Pump rate selection Commands: Go/Stop/Fill/Pump rate
Peristaltic pump (3 channels)	1	Relais board (PC-63)	Two pump rates Commands: Stop/Go
Magnetic 3/2-way valve	6	Relais board (PC-63)	Flow-channel selection
Injection valve	2	Relais board (PC-63)	Injection of sample and reagent solutions
6-way valve	2	Relais board (PC-63)	Selection between standards or determination channels
Detector[a]	1	RS 232 card A/D converter (PC- 74)	Auto-zero scaling Conversion of detector signal

[a] Fluorescence detector (F1050, Merck-Hitachi)

One 6-way valve was used to select between different standard solutions, the other to select between parallel-configured determination channels. The latter enabled the sequential determination of six different samples during one cycle of analysis. Moreover, a six fold prolongation of the sample reaction time without an essential decrease of the sample throughput as proposed by Ruzicka and Hanssen (1988) can be obtained

when the sample plugs are loaded into the channels and analyzed one after the other. Only one detector was necessary for the sequentially implemented determinations. It is also possible to combine six different detectors in serial and in parallel.

Automatic control of all active hardware components and on-line signal evaluation was performed with the software package FIACCO (Flow-Injection Analysis Control and Configuration). All valve positions and the state of the piston pumps were checked automatically, ensuring a high degree of reliability and self diagnosis. Malfunction was promptly indicated. Nearly all common FIA configurations (Ruzicka and Hanssen, 1988) for merging-zone, normal and reversed FIA procedures and stopped-flow regimes could be implemented. The exact timing and the highly constant flow rates of the piston pumps also allowed kinetic measurements, especially with respect to measurements of enzyme activities (Steube and Spohn, 1994). An optimized timetable of commands was preadjusted for every FIA procedure and was executed in a repeated manner. The timetable contained a separate time course defining the switching states for every active hardware component. The time resolution of all switching commands, including the sample and reagent injection times, was 0.5 s.

The sampling time was adapted to the required rinsing and residence times in the FIA-system and to the time delay caused by the sample system. A maximum of six different sampling procedures could be programmed, enabling automatic adaptation to the pertinent working assay.

The detector signal was scanned and saved during the complete determination cycle. The data-logging rate was one measuring point per 100 ms. Time-windows for baseline calculation, peak evaluation and signal recording were used in connection with the autozero to avoid the influence of regular disturbances in the detector signal on the peak evaluation. Such disturbances can be caused by the restarting of the piston pumps, by injection events or by switching over between different carrier solutions.

The results of an injection series for one measurement were evaluated with respect to outliers and detection limit. The latter was determined by a preselected limit of the Signal to Noise ratio (S/N). Signals with maximum

peak heights lower as this limit were ignored. To determine outliers in an injection series, the arithmetic average of the peak values in one injection series was calculated. Preselected lower and upper limits relative to the calculated average determined when the result of one injection was ignored.

The frequency of recalibration was programmed depending on the long-term stability of the determination channels. During the recalibration, two selected standard solutions were measured and the results were compared with previous measurements of these standards. When these results exceeded or fell below preselected limits, a complete calibration procedure with a maximum of six standards was repeated automatically. The calibration graph was fitted with polynomials of up to the seventh order.

The time required to develop a new determination procedure was reduced by generating a configuration file, which contained the names of the activated hardware components selected by the user. Complete FIA methods with different time courses and parameters could be copied and recalled.

Reagents and chemicals

Glucose dehydrogenase from *Bacillus megaterium* (GLCDH, EC 1.1.1.47, Merck, Darmstadt, Germany), lactate dehydrogenase from rabbit muscle (LDH, EC 1.1.1.27, Sigma, Deisenhofen, Germany), glutamic/pyruvic transaminase from *E. coli* (GPT, EC 2.6.1.2, Sigma), glutaminase from *E. coli* (GLNASE, EC 3.5.1.2, Sigma), mutarotase (EC 5.1.3.3, Sigma) from porcine kidney and glutamate dehydrogenase from bovine liver (GLTDH, EC 1.4.1.3, Sigma), were used for the enzymatic assays in the FIA system. All other chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany).

Enzyme reactors

The enzymes were immobilized and/or co-immobilized on amino-propylsilylated, controlled-pore glass with an average pore diameter of 90 nm (BIORAN G001/090/C/250, sieve fraction 180-200 µm, Schott, Mainz, Germany) according to Spohn *et al.* (1991). However, the

immobilization method for glutaminase was modified by leaving out the borohydride reduction step and decreasing the pH to 6.5 after the activation by glutardialdehyde. The immobilized enzymes were packed into Plexiglas tubes (inner diameter 3 mm) with inner threaded ends for the connecting fittings of the applied Teflon tubes (inner diameter 0.5 mm, Latek, Heidelberg, Germany). The length of the active packing was varied according to the specific enzyme activity. Polypropylene nets (20 µm hole diameter, Pharmacia, Bromma, Sweden) were placed between the packing and the fittings.

Dialysis cells

The dialysis cells consisted of two mirror-symmetrical Plexiglas plates with meander grooves (Fig. 1). The grooves had a width of 1.5 mm and a depth of 0.2 mm on the donor and the acceptor side. A regenerated cellulose membrane (cut off 10,000, Reichelt, Heidelberg, Germany) and a Teflon membrane (porosity 70 - 80 %, mean pore size 0.2 µm, thickness 20 µm, Sartorius, Heidelberg, Germany) were placed between the two plates. The effective membrane exchange area was 504 mm^2.

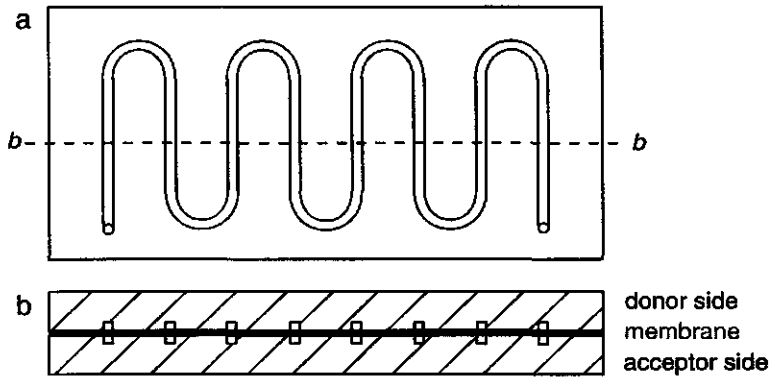

Fig. 1. One plate of the dialysis cell with the meander grooves (a). Two, symmetrical plates were separated by a membrane, whereby the donor and acceptor solutions flow co-currently through the separated grooves (b).

Off-line analysis

To control the on-line FIA measurements, samples were taken for off-line determinations. Glucose and lactate were measured with an amperometric two-channel analyzer (YSI 2000, Yellow Springs, USA). Glutamine and glutamate were measured enzymatically according to Bergmeyer *et al.* (1985a). Ammonium was measured photometrically using the reductive amination of α-oxoglutarate with NADH and glutamate dehydrogenase (Bergmeyer *et al.*, 1985b).

Animal-cell culture

A mouse/mouse hybridoma strain producing an IgG_{2a} antibody, was cultivated continuously in a fluidized-bed reactor with a total reactor volume of 170 ml. The cells were immobilized on open-porous microcarriers (Siran^R glass, Schott, Mainz, Germany) with a modified surface (Lüllau *et al.*, 1992). The perfusion rate varied between 0 - 0.45 l h^{-1}. The medium was composed of a 3:1 mixture of DMEM and Ham's F-12 (Gibco, Eggenheim, Germany) with supplements (Thömmes *et al.*, 1993). Continuous sampling was carried out with a cross-flow microfiltration module as described by Van der Pol *et al.* (1994).

RESULTS AND DISCUSSION

Application of flow-rate control

Variation of the residence time of the sample in a cartridge filled with immobilized enzyme (enzyme reactor) may be used to control the degree of substrate conversion. Elongation of the residence time of the sample in the enzyme reactor by decreasing the flow rate, will increase the degree of substrate conversion (Olsson *et al.*, 1983 and 1986). The principle of this method is shown in Fig. 2. The sample is transported with flow rate $\dot{V}_0$ to the enzyme reactor. At the moment the sample enters the enzyme reactor, the flow rate is reduced to $\dot{V}_{R,i}$, for a period of time (t_1-t_2). After this period, the flow rate is enhanced to $\dot{V}_S$, to accelerate the rinsing of the sample out of the enzyme reactor.

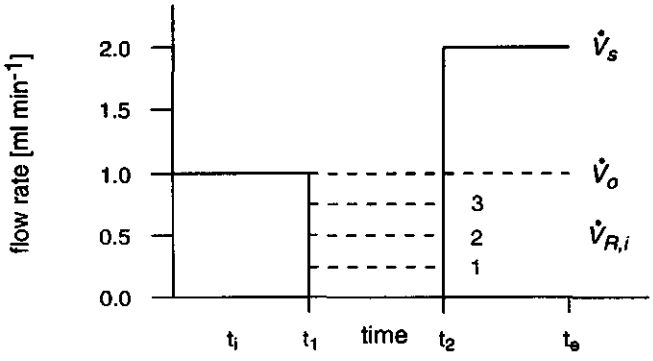

Fig. 2. Flow rate control in the FIA set-up shown in Fig. 3. $\dot{V}_0$ = starting flow rate, $\dot{V}_{R,i}$ = flow rate during the substrate conversion in the enzyme reactor and $\dot{V}_S$ = flow rate of rinsing.

The flow-rate control can be used for example, to extend the operational time of enzyme reactors. Due to inactivation during four months of operating, an enzyme reactor containing immobilized glucose dehydrogenase (GLCDH) reached 20 % conversion of the initial value in a continuous flow mode. The GLCDH-reactor was used to determine the glucose concentration with the FIA set-up shown in Fig. 3.

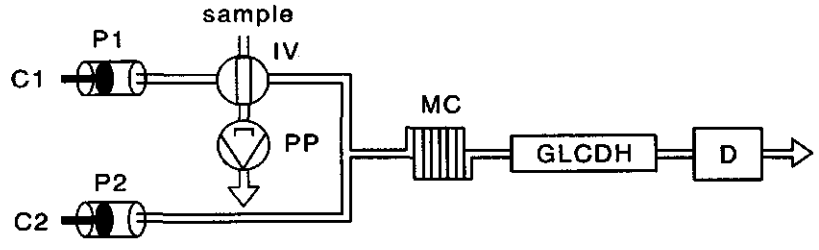

Fig. 3. FIA set-up for the enzymatic determination of glucose. GLCDH = glucose dehydrogenase reactor, IV = injection valve, C1-2 = carrier solutions, P1-2 = piston pumps, PP = peristaltic pump, MC = mixing coil, D = detector.

To improve the conversion, the flow rate in the enzyme reactor was decreased. Fig. 4 shows the dependence of the maximum peak height on the flow rate $\dot{V}_{R,i}$ for a 20 s and 40 s period of decreased flow rate, respectively. The flow rate $\dot{V}_{R,i}$ was adjusted automatically at the moment the sample/reagent zone entered the enzyme reactor. It was noticed that flow rates smaller than 0.08 ml min^{-1} resulted in a higher maximum peak height than when the flow was totally stopped for the same time period. This could be due to a convective mass transfer in addition to the diffusional mass transfer during a complete stoppage of the flow. Obviously, longer periods with zero or reduced flow rates increased the maximum peak height. Manipulating the conversion by means of the flow rate can also be used to adapt the sensitivity of the assay to different concentration ranges without changing the dispersion factor.

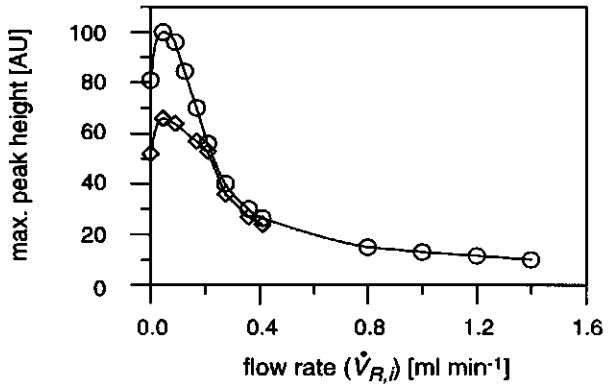

Fig. 4. Dependence of the maximum peak height in arbitrary units [AU] on the flow rate during decreased flow rate times of 20 s (◇) and 40 s (○) in the enzyme reactor. A 0.25 mM glucose solution was injected (20 µl) into the FIA-manifold shown in Fig. 3.

The reduced-flow method can also be used to achieve 100 % conversion of the analyte in the enzymatic reactor. The resulting increased reaction times are counterbalanced by a lower sensitivity of the assay to

factors which influence the conversion efficiency, such as enzyme effectors, temperature and variations in the flow rate (Johansson *et al.*, 1983). An extended linear determination range can be achieved with 100 % conversion in addition.

Another application of the reduced-flow technique is the control of the degree of transfer of an analyte across a membrane in a dialysis cell (separation efficiency). Dialysis not only dilutes the sample but also separates the analyte from unwanted components in the sample, i.e. those interfering components,. Fig. 5 shows the separation efficiency of glucose (2 mM) across a membrane of regenerated cellulose in relation to the time periods of stopped-flow for both the donor and acceptor flow. The glucose solution was dialyzed before injection, whereby the zone of glucose in the acceptor solution obtained during the stopped-flow period (dialysate) was injected into a FIA-configuration similar to Fig. 3. The separation efficiency was calculated from the maximum peak height ratio between the injection of the dialysate and the non-dialyzed sample solution.

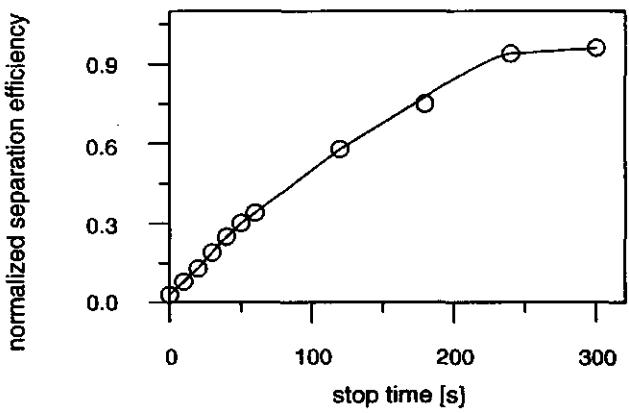

Fig. 5. Dependence of the separation efficiency on the stop time in the dialysis cell for a 2 mM glucose solution. Both the donor and acceptor flow entered at the same side of the dialysis cell (co-current). The flow rates were 2 ml min^{-1}, except for the stopped-flow periods. A normalized separation efficiency of 1.0 is equal to 50 % of the maximum peak height of the injection of a non-dialyzed glucose solution.

Sequential enzymatic determination of a volatile and a non-volatile analyte

Gas dialysis and enzymatic assays could be combined in the FIA-system to measure volatile and non-volatile substances simultaneously with improved selectivity. The enhancement of the selectivity by gas dialysis was used to determine simultaneously ammonium and glutamate. The analytical set-up is shown in Fig. 6. Both glutamate and ammonium were measured using immobilized glutamate dehydrogenase.

For the ammonium determination, the sample was injected into the carrier C3, which consist of 0.1 M NaOH to convert ammonium to ammonia with a degree > 99.9 %. The ammonia formed was separated from the glutamate in a thin-layer gas dialysis cell containing a hydrophobic Teflon-membrane. The acceptor solution (C4) consisted of 0.1 M tris-(hydroxymethyl)-aminomethane (TRIS), pH 8.0, 20 mM α-ketoglutarate and 0.2 mM NADH.

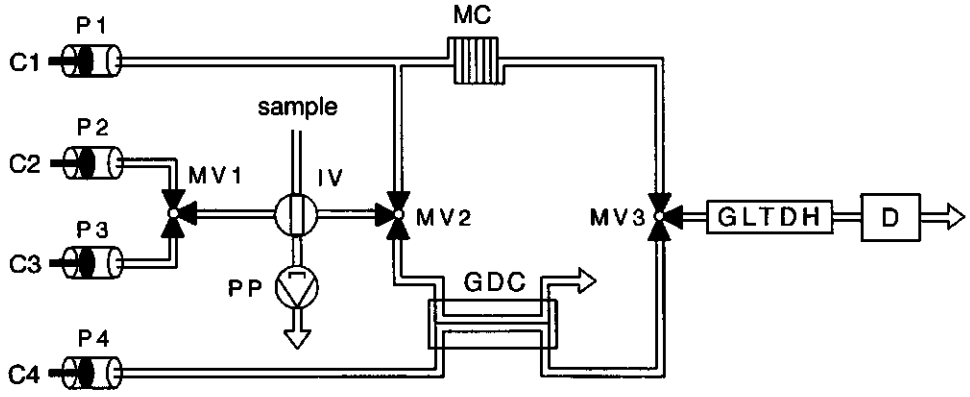

Fig. 6. FIA set-up for the sequential, enzymatic determination of ammonium and glutamate. The glutamate dehydrogenase reactor was 3 cm long and the injection volume was 10 µl for both determinations. GLTDH = glutamate dehydrogenase reactor, GDC = gas dialysis cell, PP = peristaltic pump, MV1-3 = 3/2-way valves, IV = injection valve, MC = mixing coil, P1-4 = piston pumps, C1-4 = carrier solutions and D = detector.

The dialyzed ammonia/ammonium was consumed in the reductive amination of α-ketoglutarate according to:

$$\alpha\text{-Ketoglutarate} + NADH + NH_4^+ \rightleftharpoons \text{Glutamate} + NAD \quad (1)$$

catalyzed by the glutamate dehydrogenase, whereby NADH was detected fluorometrically. Since NADH was consumed and not produced, the signal of the detector was reversed electronically to enable peak evaluation. Ammonium could be determined in the range from $C = 0.002$ mM to 1 mM. In this range, the least squares, linear regression line was:

$$Y = [1.01\ (\pm\ 0.07)\ \text{l mmol}^{-1}]\ C + 0.06\ (\pm\ 0.05) \quad (2)$$

whereby Y represents the "reversed" maximum peak height and C the ammonium concentration (mM). The correlation coefficient was $r^2 = 0.996$ ($n = 5$), with a level of insignificance of $\alpha = 0.05$. The normalized "reversed" maximum peak height $Y = 1$ corresponds to 1 mM ammonium. Glutamate concentrations of up to 20 mM in the sample solution had no significant influence on the ammonium determination.

For the glutamate determination, the carrier solutions C3 and C4 were replaced by the solutions C2 and C1 by switching simultaneously pumps P3 and P4 off and pumps P2 and P1 on. Moreover, the 3/2-way valves MV1-3 were switched over. Consequently, the sample solution was injected into solution C2, containing 0.1 M TRIS and 0.1 M Na_2SO_4, which was adjusted to pH 9.5. The carrier solution C2 was mixed with solution C1 containing 2.5 mM NAD and 25 mM $(NH_4)_2SO_4$ in 0.1 M TRIS buffer adjusted to pH 6.5. This relatively high ammonium concentration was used to suppress the influence of small amounts of ammonium in the sample. The analytical reaction is the reverse reaction of Eq. 1, whereby glutamate is deaminated to α-ketoglutarate producing NADH. The least squares, linear calibration equation was:

$$Y = [2.00\ (\pm\ 0.04)\ \text{l mmol}^{-1}]\ C + 0.07\ (\pm\ 0.03) \quad (3)$$

where Y represents the maximum peak height and C the glutamate concentration. The correlation coefficient was $r^2 = 0.997$ ($\alpha = 0.05$, $n = 4$) for the concentration range 0.05 - 0.5 mM glutamate. The normalized maximum peak height $Y = 1$ corresponds to 0.5 mM glutamate. When ammonium (1 mM) was present in the sample, the least squares, linear calibration equation for the same range became:

$$Y = [1.99\ (\pm\ 0.05)\ \mathrm{l\ mmol^{-1}]\ } C + 0.03\ (\pm\ 0.03) \qquad (4)$$

with $r^2 = 0.997$ ($\alpha = 0.05$, $n = 4$). A maximum of 1.5 mM ammonium in the sample did not significantly change the regression line.

Combination of a chemical and enzymatic assay in one FIA-manifold

The enzymatic determination of ammonium using glutamate dehydrogenase (Eq. 1) is not ideal as it produced reversed peaks and the required NADH solution is relatively unstable and expensive. Therefore, a non-enzymatic procedure was developed. Several authors applied the reaction of ammonia with o-phthaldialdehyde (OPA) in the presence of mercaptoethanol and a partial non-aqueous solution (Roth, 1971; Aoki *et al.*, 1983; Garcia Alvarez-Coque *et al.*, 1989). Since OPA reacts with all amino groups present in a sample, amino acids will interfere in most cases. This interference can be avoided by separation of ammonium from the sample by gas dialysis over a hydrophobic membrane. The non-aqueous OPA solution however hydrophilises the membrane resulting in leakage of the donor solution containing amino acids, into the acceptor solution (OPA). An OPA reagent solution without organic solvents was therefore developed. Replacing the mercaptoethanol with thioglycolic acid allowed the preparation of a totally aqueous OPA reagent with an acceptable stability of approximately 14 d. The reagent solution consisted of 7.5 mM OPA and 28.7 mM thioglycolate in 0.4 M borate buffer, pH 10.4. Ammonia reacts with OPA according to:

$$NH_3 + \mathrm{OPA} + \mathrm{Thioglycolate} \rightleftharpoons \mathrm{Isoindol\text{-}derivative} \qquad (5)$$

whereby the isoindol-derivative was measured fluorometrically at the same wavelengths (excitation 340 nm, emission 440 nm) as NADH.

The determination of ammonium using gas dialysis and the newly developed OPA reagent was combined with the enzymatic determination of glucose in a FIA set-up similar to Fig. 6, whereby the glutamate dehydrogenase reactor was replaced by a glucose dehydrogenase reactor. The enzyme reactor was positioned before valve MV3. The aqueous standard solutions were injected into a carrier solution (C3), which consisted of 0.1 M TRIS, pH 10.6 and 20 mM Na_2SO_4. The sample was directed through the gas dialysis cell (GDC) for the ammonium determination. The acceptor solution (C4) was the OPA reagent. The least squares, linear regression line for the concentration range 0.1 - 2.0 mM ammonium was determined as:

$$Y = [2.695 \pm (0.046)\ \mathrm{l\ mmol^{-1}}]\ C + 0.187\ (\pm 0.095) \qquad (6)$$

with $r^2 = 0.998$ ($\alpha = 0.05$, $n = 5$).

To measure glucose, the magnetic valves MV2 and MV3 were switched over. Valve MV3 was used to prevent leakage of the OPA reagent into the enzyme reactor. The piston pumps P4 and P1 were switched synchronously off and on, respectively. The carrier solutions C3 and C1, which consisted of 2.5 mM NAD and 0.1 M TRIS, pH 1.6, were consequently mixed in the mixing coil MC. The resulting pH of 7.3 after mixing was the optimum pH for the glucose dehydrogenase. The low pH value of carrier solution C1 kept the NAD solution stable for more than 8 weeks. Since it was found that Na^+-ions activate glucose dehydrogenase in TRIS buffer solutions (data not shown), Na_2SO_4 was added to carrier C3. Glucose could be determined in the range 0.2 - 1.5 mM on the basis of the regression line:

$$Y = [3.136\ \mathrm{l\ mmol^{-1}}\ (\pm 0.062)]\ C + 1.05\ (\pm 2.53) \qquad (7)$$

with $r^2 = 0.999$ ($\alpha = 0.05$, $n = 5$). To implement both determination procedures in the same detector range and with one and the same parameter set, a wide-range autozero was necessary due to the high basis signal of the OPA reagent in comparison with NAD. After switching from the enzymatic

to the non-enzymatic assay, the baseline of the detector shifted out of range, which was caused by the high fluorescence of the OPA reagent alone. The automatic autozero enabled the back-scaling of the fluorescence signal to the primary scale. Fig. 7 demonstrates the effect of a two-fold recall of the autozero for the fluorimetric determination of ammonium. The first autozero call set the baseline to its original level (t_{AZ1}). After further 10 s, the autozero was repeated (t_{AZ2}).

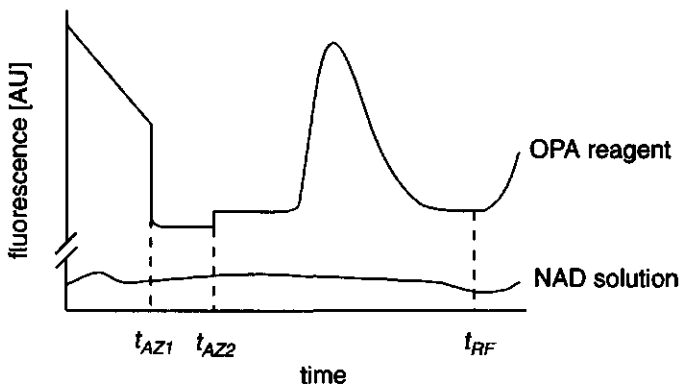

Fig. 7. Course of the detector signal in arbitrary units (AU) during the determination of ammonium with the OPA method in comparison to the baseline of a 1.25 mM NAD solution in 0.1 M TRIS buffer. t_{AZ1}, t_{AZ2} = first and second autozero time, t_{RF} = start of refilling of piston pumps.

Sequential determination of five analytes

Ammonium, glucose, glutamine, glutamate and lactate are important key-parameters for animal-cell cultures. Glucose, glutamine, glutamate and lactate can be determined enzymatically by means of immobilized dehydrogenases (Table 2). The advantage of the dehydrogenases is their independence of the oxygen concentration in contrast to the also applicable oxydases.

Table 2. Conversion of glucose, glutamine, glutamate and lactate with dehydrogenases in combination with glutaminase (Eq. 9) and glutamic/pyruvic transaminase (Eq. 12).

Glucose + NAD	$\longrightarrow$	Gluconate + NADH	(8)
Glutamine + H_2O	$\longrightarrow$	Glutamate + NH_4	(9)
Glutamate + NAD	$\rightleftharpoons$	α-Ketoglutarate + NADH + NH_4	(10)
Lactate + NAD	$\rightleftharpoons$	Pyruvate + NADH	(11)
Pyruvate + Glutamate	$\rightleftharpoons$	Alanine + α-Ketoglutarate	(12)

The above described determination of ammonium with OPA reagent and the enzymatic determinations for the other key-components were implemented into one FIA set-up (Fig. 8). The NADH and isoindol-derivative produced were detected fluorometrically at the same wavelengths. Substances, which are easy oxidized as ascorbic acid, uric acids and iron (II), did not interfere with the proposed determination procedures. However, the optimum pH conditions for the four enzymatic assays were not compatible with each other. Therefore, two carrier solutions with pH 10.6 (C1) and pH 1.6 (C2 or C3) were mixed in different ratios to adjust to the optimal pH for each assay. The pH could be precisely adjusted between 6.5 - 8.5 by mixing these two carriers (Fig. 9).

The immobilized glutaminase (Eq. 9, Table 2) has a pH optimum of approximately 4.9 and is stable between pH 4.5 - 6.0, whereas glutamate dehydrogenase (Eq. 10, Table 2) has a pH optimum of approximately 8.4. Therefore, glutamine had to be determined in two sequential steps. The glutamine determination started with continuous dialysis of the sample against a 0.05 M sodium acetate buffer with pH 4.9. The flow rates of both the sample and acceptor solution were 1 ml min^{-1}. The acceptor solution was directed over a reactor containing immobilized glutaminase (GLNASE), resulting in the formation of glutamate from glutamine. The sample solution containing the converted glutamine was injected into carrier C1, which was mixed with carrier C2 to adjust the optimum pH required for the glutamate dehydrogenase. Carrier solution C1 consisted of 0.1 M TRIS and 20 mM Na_2SO_4 and C2 consisted of 0.1M TRIS, 2.5 mM NAD.

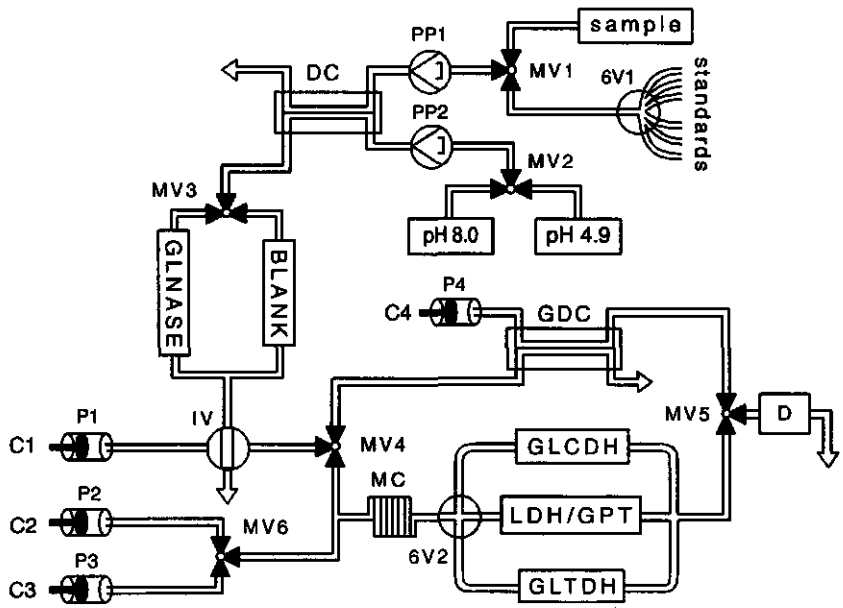

Fig. 8. FIA set-up for the automatic determination of ammonium, glucose, lactate, glutamine and glutamate (see text). Enzyme reactors: GLCDH = glucose dehydrogenase, LDH/GPT = lactate dehydrogenase/glutamic/ pyruvic-transaminase, GLTDH = glutamate dehydrogenase and GLNASE = glutaminase. BLANK = blank reactor (see text), DC = dialysis cell, GDC = gas dialysis cell, MC = mixing coil, PP1-2 = channels of peristaltic pump, P1-4 = piston pumps, C1-4 = carrier/reagent solutions, MV1-6 = 3/2-way valve, 6V1-2 = 6-way valves, IV = injection valve and D = detector.

To compensate for glutamate present in the sample, this analyte was measured separately by bypassing the glutaminase reactor (valve MV3). A blank reactor, containing only controlled-pore glass carriers, was integrated into the by-pass to achieve a similar dispersion to that in the glutaminase reactor. The difference between the peak areas was used to determine the glutamine concentration. For all other determinations, the sample solution was dialyzed against a 0.1 M TRIS buffer, pH 8.0, by switching valve MV2.

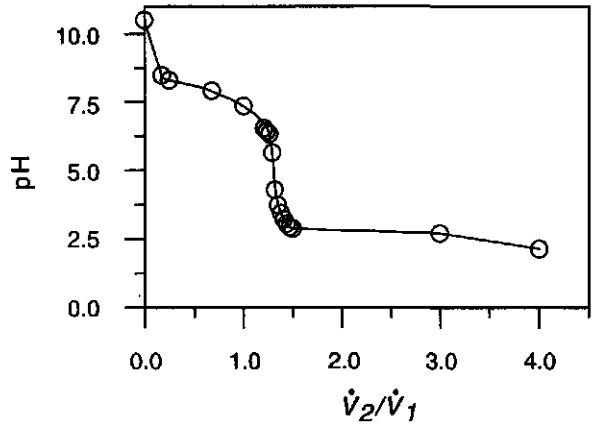

Fig. 9. pH-working curve for mixing buffer solution C2, pH 1.6, with buffer solution C1, pH 10.6. $\dot{V}_2$ and $\dot{V}_1$ = pertinent flow rates.

To implement the lactate determination according to Eq. 11 and Eq. 12 (Table 2), carrier solution C2 was replaced by another carrier solution (C3) to supply the necessary glutamate. Carrier solution C3 consisted of 1.0 M NaCl, 5 mM NAD and 30 mM sodium glutamate in 0.1 M TRIS, pH 1.6, and was mixed with the carrier C1 to adjust the optimum pH value of 8.0.

The adaptation of all determination procedures to the same fluorescence detector range was achieved by adjusting the flow rates of the carrier solutions and the corresponding residence times in the dialysis cell, the gas dialysis cell and the enzyme reactors (Table 3).

The FIA system was tested for the fast sequential determination of glucose, lactate, glutamine, glutamate and ammonium in mixed standard solutions, which were adapted to real cell-culture media with respect to the expected concentration ranges and the protein content. Table 4 summaries the determination ranges, the regression line equations and the Maximum Injection Rates (MIR).

Table 3. Parameters for the determinations using the FIA set-up shown in Fig. 8.

	Flow C1, pH 10.6 ml min^{-1}	Flow of second carrier, pH 1.6 ml min^{-1}	Stop time in enzyme reactor s	Stop time in dialysis cell s
Glucose	1.50	C2 = 1.50	0	0
Lactate	1.50	C3 = 0.50	0	10
Glutamine	0.75	C2 = 0.25	20	30
Glutamate	0.75	C2 = 0.25	20	30
Ammonium	0.50	C4 = 0.50	-	30

Table 4. Calibration curves for the five-channel-FIA shown in Fig. 8.
(6 data points, $\alpha = 0.05$, n = 4)

Glucose

$Y_1 = [1.008\ l\ mmol^{-1}\ (\pm 0.012)]\ X_1 + 0.015\ (\pm 0.019)$ (13)

$Y_1 = 1$ for $C_1 = 50$ mM with $X_1 = C_1 / (50\ mM)$,

range: $X_1 = \langle 0.1;50 \rangle$ mM, $r^2 = 0.999$; MIR = 100 h^{-1}

Lactate[a]

$Y_2 = [0.992\ l\ mmol^{-1}\ (\pm 0.015)]\ X_2 + 0.024\ (\pm 0.020)$ (14)

$Y_2 = 1$ for $C_2 = 20$ mM with $X_2 = C_2 / (20\ mM)$

range: $X_2 = \langle 0.1;20 \rangle$ mM, $r^2 = 0.996$; MIR = 20 h^{-1}

Glutamine

$Y_3 = [0.994\ l\ mmol^{-1}\ (\pm 0.011)]\ X_3 + 0.009\ (\pm 0.019)$ (15)

$Y_3 = 1$ for $C_3 = 5$ mM with $X_3 = C_3 / (5\ mM)$

range: $X_3 = \langle 0.1;\ 5 \rangle$ mM, $r^2 = 0.998$; MIR = 50 h^{-1}

Glutamate

$Y_4 = [0.999\ l\ mmol^{-1}\ (\pm 0.012)]\ X_4 + 0.010\ (\pm 0.018)$ (16)

$Y_4 = 1$ for $c_4 = 5$ mM with $X_4 = C_4 / (5\ mM)$

range: $X_4 = \langle 0.1;\ 5 \rangle$ mM, $r^2 = 0.999$; MIR = 50 h^{-1}

Ammonium

$$Y_5 = [1.005\ l\ mmol^{-1}\ (\pm\ 0.016)]\ X_5 + 0.034\ (\pm\ 0.021) \qquad (17)$$

$Y_5 = 1$ for $C_5 = 20$ mM with $X_5 = C_5 / (20\ mM)$

range: $X_5 = <0.1; 20>$ mM, $r^2 = 0.992$; MIR = 60 h^{-1}

[a] with co-immobilized GPT/LDH

The reliability and the robustness of the 5-channel configuration was tested during several runs lasting 120 h. Table 5 shows the decrease of the measured maximum peak heights with time. All enzyme reactors appeared to be stable enough to monitor animal-cell cultures for more than 5 d. The stability of the lactate channel was determined by the stability of the immobilized glutamic/pyruvic transaminase. However, in the desired concentration range from 0.1 - 20 mM, lactate may be also determined with immobilized lactate dehydrogenase alone. This allows a three fold increase in the injection rate. Applying only lactate dehydrogenase gave a parabolic calibration curve, which enabled lactate determinations with a relative standard deviation ranging between 1.1 - 2.3 % ($\alpha = 0.05$, $n = 4$).

Table 5. Stability of the enzyme reactors and of the OPA reagent, T = 15 °C, determined with FIA set-up shown in Fig. 7. Sensitivity is 100 % at time = 0 h.

Reactor/reagent	Analyte concentration	Relative sensitivity (%) after	
	mM	48 h	120 h
Glutaminase[a]	3.0	98	70
GLTDH	3.0	97	72
GLCDH	5.0	85	70
LDH/GPT	2.5	90	55
OPA	2.5	99	97

[a] OPA fluorimetric detection of the released ammonium. GLTDH = glutamate dehydrogenase, GLCDH = glucose dehydrogenase, LDH/GPT = lactate dehydrogenase/glutamic/pyruvic transaminase.

Although the FIA set-up described above proved its usefulness for the on-line monitoring of animal cultures, several improvements could be made to enhance the robustness and sample frequency. Since fouling of the dialysis membrane by proteins present in the sample could cause errors and disturbances during long-term cultures, the dialysis cell was placed behind the injection valve. This resulted not only in a negligible membrane fouling but also in faster response times. However, the pulsed dialysis decreased the sensitivities of the determinations around 10 times in comparison to the above described set-up. This could be compensated by increasing the sensitivity of the detector. Since it was also found that the glutaminase reactor was more stable in a pulsed FIA mode, this reactor and the blank reactor were also placed behind the injection valve. The modified FIA set-up is shown in Fig. 10. A further modification was made by changing the pH of the carrier solutions (Table 6) to avoid complete inactivation of the immobilized enzymes during disturbances in the FIA-manifold. Moreover, sodium azide was added to prevent microbial growth. All methods used for the determinations were the same as described for the FIA set-up shown in Fig. 8, except that lactate was determined without glutamic/pyruvic transaminase, and the analyses were done without stopped-flow steps.

The standard solutions for calibration were prepared in cell-culture medium to minimize the influence of interfering substances. The measurement of the five compounds with double injections for glucose, lactate and glutamate and triple injections for glutamine and ammonium, and rinsing the sampling tube with 0.2 M sulfuric acid for 5 min, took a total of 42 min.

Table 6. Composition of the carrier solutions for the on-line monitoring of animal-cell cultures with the FIA-configuration shown in Fig. 9.

Carrier	Composition	pH
C1	0.2 M TRIS, 0.2 M Na_2SO_4, 0.02 % (w/v) NaN_3	7.15
C2	0.2 M TRIS, 0.2 M Na_2SO_4, 0.02 % (w/v) NaN_3	9.00
C3	0.2 M TRIS, 0.2 M Na_2SO_4, 2.5 mM NAD, 0.02 % (w/v) NaN_3	5.00
C4	OPA reagent	10.40

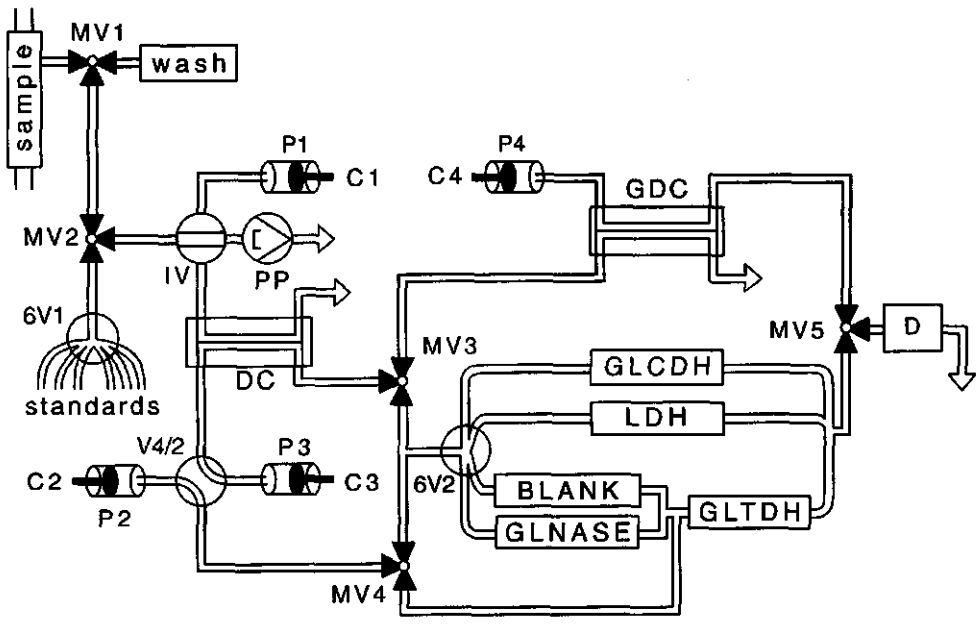

Fig. 10. FIA set-up for long-term, on-line monitoring of animal-cell cultures (see text). Enzyme reactors: GLCDH = glucose dehydrogenase, LDH = lactate dehydrogenase, GLTDH = glutamate dehydrogenase and GLNASE = glutaminase. BLANK = blank reactor (see text), DC = dialysis cell, GDC = gas dialysis cell, PP = peristaltic pump, P1-4 = piston pumps, C1-4 = carrier/reagent solutions, MV1-5 = 3/2-way valves, 6V1-2 = 6-way valves, IV = injection valve, V4/2 = 4/2-way valve and D = detector.

Fig. 11 shows the course of the on-line measured concentrations of ammonium, glutamine, lactate and glucose during the start-up period of a hybridoma culture. The glutamate concentration appeared to be very low and did not change significantly, it is therefore not mentioned further. The animal-cell culture was monitored on-line without interruptions during the first 130 h. The off-line and the on-line measured concentrations correspond well for glucose, glutamine and ammonium. The on-line monitored lactate values were significantly lower than the off-line values, which were measured by the amperometric lactate sensor.

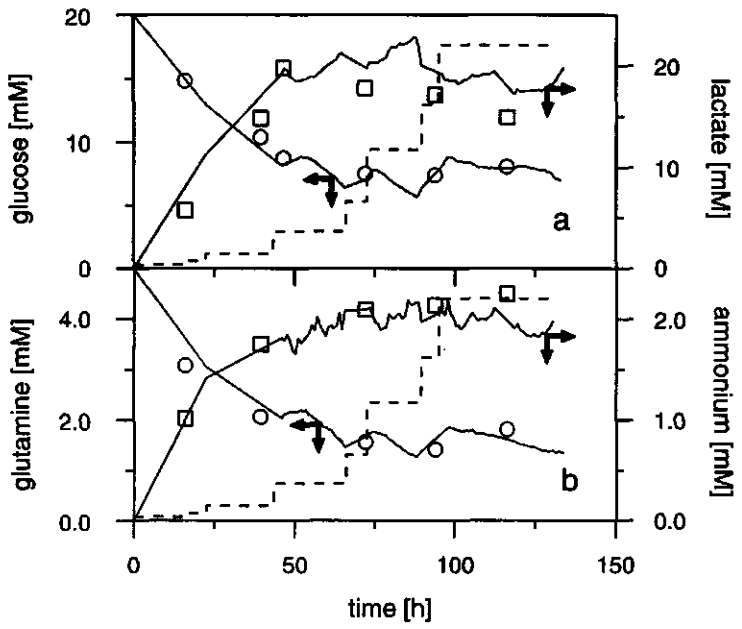

Fig. 11. On-line monitored concentrations during the start-up of a continuous animal-cell culture. The lines represent data of the FIA-manifold. The broken lines represent the dilution rate (D_{max} = 0.44 h^{-1}). (a) Off-line data of glucose (O) and lactate (□), (b) off-line data of glutamine (O) and ammonium (□).

The long-term stability of the enzymatic assays was tested under process conditions for 55 d in a separate experiment. Fig. 12 shows the measured maximum peak heights for the injection of 0.34 g l^{-1} glutamine, 1.8 g l^{-1} glucose and 1.8 g l^{-1} lactate under the conditions of on-line process monitoring. Up to 3000 on-line determinations with the lactate and glucose assay and up to 4500 on-line determinations with the glutamine assay could be done without exchanging the enzyme reactors. The long-term stability of the FIA system may be further improved by two equal enzyme reactors for the most frequently used determination channels.

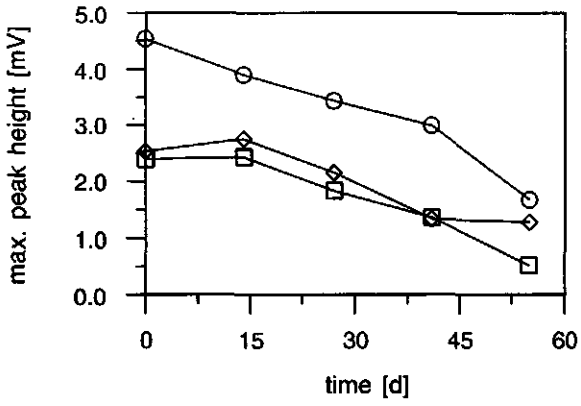

Fig. 12. Stability of the enzymatic determination channels for glucose (○), glutamine (□) and lactate (◇) during the on-line monitoring of an animal-cell culture.

Other, more detailed investigations of animal-cell cultures using the FIA-system are described by Van der Pol *et al.* (1994). Attempts to use the proposed FIA set-up to control automatically the glucose and the glutamine concentrations during a continuous cell culture are in progress.

CONCLUSIONS

A multi-channel FIA-system has been realized by the development of a robust and flexible software program, which could control a large amount of hardware components. The software program also carried out peak evaluation, a simple outlier test, and calculated detection limits and calibration curves. The independent control of every hardware component with a time resolution of 0.5 s, allowed a variety of FIA methods to be implemented to adjust the optimum reaction conditions and detection range for every assay. The latter was mainly achieved by fine tuning of the residence time, using flow rate control and/or stopped flow. The optimum pH of every assay was adjusted by mixing two carrier flows in different ratios.

Dialysis and gas dialysis were applied to dilute the sample and to separate the analyte from interfering compounds present in the sample. For example, gas dialysis allowed the determination of ammonium with an aqueous OPA reagent in the present of other amino groups, e.g. amino acids.

Chemical and enzymatic assays could be combined in the FIA set-up for the on-line monitoring of five components during continuous animal-cell cultures. A pre-requisite for the success of the long-term monitoring was the application of precision pumps and reliable and robust hardware components in the FIA-manifold. On-line monitoring of other bioprocesses than animal-cell cultures will be also possible with this newly developed analytical system as a result of its high flexibility.

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Chapter 3

On-line Monitoring of an Animal-Cell Culture with Multi-Channel Flow-Injection Analysis

SUMMARY

A multi-channel flow-injection analysis system was used for on-line monitoring of a continuous animal-cell culture with high cell density. With this system, the glucose, lactate and glutamine concentrations were determined using immobilized dehydrogenases. The ammonium concentration was measured by using an aqueous o-phthaldialdehyde solution. The glutamine concentration was determined on the basis of the difference between a glutamine and a glutamate measurement. To prevent disturbance of the measurement and pollution of the system, the analytes in the sample were separated from high-molecular compounds by on-line dialysis. On-line gas dialysis was used to avoid interference of other amino groups with the ammonium determination. In addition, dialysis was used as a dilution step. The measurement time for all four components was 42 min. This time period included a final washing period after the analysis cycle. The system was calibrated once a day.

Two continuous cultures of a hybridoma cell line immobilized in open-porous glass carriers were monitored, using a fluidized-bed reactor as culture system. The concentration of glutamine, glucose and ammonium determined with the on-line FIA-system were in good agreement with the off-line data determined once a day. Only the lactate data showed some deviation. The immobilized enzyme reactors could be used for up to 3000 - 5000 injections.

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During the first culture, lasting 200 h, the start-up period of the reactor was monitored. The on-line measurements described much better the time course of the concentrations than the off-line data. It was possible to estimate the growth rate of the cells in the microcarriers by the on-line data. In the course of the second culture, which lasted almost 1000 h, the influence of the dissolved-oxygen concentration on the cell metabolism was monitored. It was noted that a sudden change of the glutamine concentration in the feed caused a fast change of the consumption and production rate of the measured metabolites.

INTRODUCTION

Flow-injection Analysis (FIA) has been widely used as a tool for the automation of determinations in the last nineteen years after the first publication by Ruzicka and Hansen (1975). The combination of dilution steps and many separation procedures, as dialysis and gas dialysis, with the use of selective biomolecules, e.g. enzymes and antibodies (Schmid and Kuennecke, 1990), makes FIA very suitable to analyse substances within a complex matrix. FIA is therefore very useful in the analysis of environmental samples, food and blood samples and samples from biotechnological processes which contain many different substances.

Publications describing on-line monitoring of biotechnological processes are mainly concentrated on the measurement of glucose (Brooks *et al.*, 1991). In most of these analysis systems, glucose oxidase is used for the measurement of glucose. The detection occurs by measuring the oxygen depletion (Holst *et al.*, 1988), the produced peroxide in various ways (Garn *et al.*, 1989; Leung *et al.*, 1991; Valero *et al.*, 1990), or using p-quinone as hydrogen acceptor (Geppert and Asperger, 1987). All these analysis devices have been tested for a period varying from a couple of hours up to 180 h, which is in general long enough for batch cultures, but not for fed-batch or continuous processes. In the case of long culture times, the stability of all analytical compounds, software and hardware is very important. Moreover, the sterility of the analytical set-up for the process analysis has to be guaranteed.

Several authors have described on-line monitoring of animal-cell cultures. The important substances besides the secreted protein, are glucose and glutamine, the main C- and N source, and their metabolites lactate and ammonium. Favre *et al.* (1990) used an HPLC for the on-line measurement of the alanine, glutamine, lactate and glucose concentration. The maximum sample frequency of the system was one injection per hour. Romette (1987) used three biosensors to measure the lactate, glutamine and glucose concentration, based on immobilized oxidases measuring the oxygen consumption. However, the achieved linear ranges for lactate (0 - 5 mM) and glutamine (0 - 1 mM) were not sufficient for animal-cell cultures. Renneberg *et al.* (1991) also used three biosensors for the determination of the glucose, lactate and glutamine concentration. The oxidases were immobilized on amperometric sensors measuring the produced peroxide. Yet, the serial configuration of two sensors could cause interference of peroxide coming from the upstream sensor. The system was calibrated for every measurement, with a sample frequency of 2 d^{-1}. Meyerhoff *et al.* (1993) developed a two-channel system, measuring the glucose and glutamine concentration in animal-cell culture medium. The glucose concentration was measured with an amperometric biosensor, using immobilized glucose oxidase. After elimination of endogenous ammonium through dialysis, glutamine was hydrolysed and the formed ammonium was measured potentiometrically. The dialytic elimination of ammonium was performed with a cationic exchange membrane. Adsorption of proteins on the electrically charged membrane could, however, occur during long time of operation. This would influence the mass transfer across the membrane.

The multi-channel flow-injection system presented in this paper was developed for on-line monitoring of prolonged cultures of animal cells. A combination of enzymatic assays with a chemical assay was used in one manifold with one fluorescence detector. The glucose, lactate and glutamine concentration were measured with immobilized enzymes. The ammonium concentration was measured chemically, using an aqueous o-phthaldialdehyde (OPA) reagent. The system was tested during two continuous cultures of hybridoma cells in a fluidized-bed reactor, lasting 200 h and 1000 h, respectively.

MATERIAL AND METHODS

Reagents

NAD and glucose dehydrogenase (EC 1.1.1.47) from *Bacillus megaterium* were obtained from Merck (Darmstadt, Germany). Lactate dehydrogenase (EC 1.1.1.27) type XI from rabbit muscle, glutamate dehydrogenase (EC 1.4.1.3) type III from bovine liver, glutaminase (EC 3.5.1.2) grade V from *Escherichia coli* and mutarotase (EC 5.1.3.3) from porcine kidney were products of Sigma (Deisenhofen, Germany). All other chemicals were analytical grade. Buffer solutions were prepared with distilled/de-ionized water.

Analytical methods

Immobilized dehydrogenases were used to determine glucose, lactate and glutamine. The formed NADH was detected fluorometrically, using the maximum peak height to calculate the concentrations.

The glucose concentration was measured using co-immobilized mutarotase (Eq. 1) and glucose dehydrogenase (Eq. 2) (Roehrig *et al.*, 1983):

$$\alpha\text{-D-Glucose} \rightleftharpoons \beta\text{-D-Glucose} \quad (1)$$

$$\beta\text{-D-Glucose} + \text{NAD} \rightleftharpoons \text{D-Gluconate} + \text{NADH} \quad (2)$$

The lactate concentration was measured using immobilized lactate dehydrogenase (Yao *et al.*, 1982):

$$\text{Lactate} + \text{NAD} \rightleftharpoons \text{Pyruvate} + \text{NADH} \quad (3)$$

Glutamine was first hydrolysed to glutamate using immobilized glutaminase:

$$\text{Glutamine} + H_2O \rightleftharpoons \text{Glutamate} + NH_4 \quad (4)$$

Glutamate was further converted to α-ketoglutarate and NADH in a second enzyme reactor, using immobilized glutamate dehydrogenase (Schelter-Graf *et al.*, 1984):

$$\text{Glutamate} + \text{NAD} + H_2O \rightleftharpoons \alpha\text{-Ketoglutarate} + NH_4 + \text{NADH} \qquad (5)$$

A chemical method was developed for the determination of the ammonium concentration. An aqueous solution of o-phthaldialdehyde (OPA), which was stable for two weeks, was used as reagent. To prevent interference of other components like amino acids, ammonium was converted to ammonia by rising the pH and separated from other components by gas dialysis. The diffused ammonia reacts with the OPA (Aoki *et al.*, 1983):

$$NH_3 + \text{OPA} + \text{Thioglycolate} \rightleftharpoons \text{Isoindol (derivative)} \qquad (6)$$

The formed isoindol derivative was measured fluorometrically at the wavelengths used for NADH determination.

The FIA-manifold

The multi-channel FIA-manifold is based on the system described by Spohn *et al.* (1994), using the same hardware. The flow diagram of the FIA-manifold is shown in Fig. 1.

The system consisted of two pneumatically actuated injection valves, two pneumatically actuated six-port valves (Rheodyne, Cotrati, USA) and five magnetic 3/2-way valves (AKZO, Ratingen, Germany). One injection valve was configurated as a 4/2-way valve. Four low pulse piston pumps (Dosimat 665, Metrohm, Herisau, Switzerland) propelled the carrier or reagent solutions through the system. The sample was sucked continuously by a peristaltic pump (Watson Marlow, Falmouth, GB) with a flow rate of 0.25 ml h^{-1}. The excitation wavelength of the fluorescence detector (Shimadzu FLD/6A) was set to 340 nm, the emission wavelength to 435 nm.

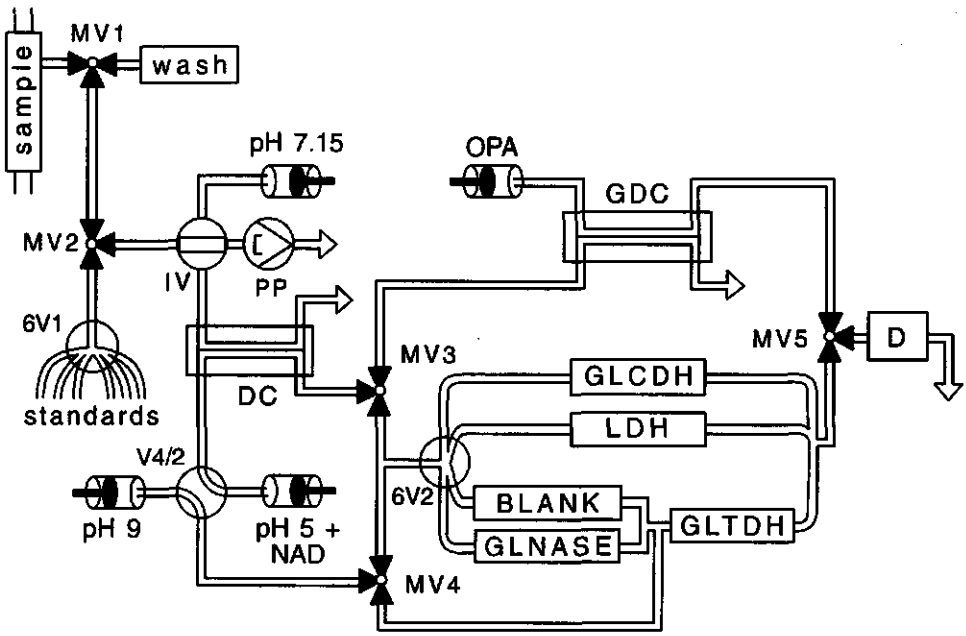


Fig. 1. Flow diagram of the flow-injection analysis manifold. MV1-5 = magnetic 3/2-way valves, 6V1-2 = pneumatic six-port valves; V4/2 = pneumatic 4/2-way valve, IV = injection valve, DC = dialysis cell, GDC = gas dialysis cell, PP = peristaltic pump. Enzyme reactors: GLCDH = glucose dehydrogenase, LDH = lactate dehydrogenase, GLNASE = glutaminase, GLTDH = glutamate dehydrogenase, BLANK = only carrier material.

A dialysis cell and a gas dialysis cell were made of Lucite and had an exchange area of 504 mm^2 (Spohn *et al.*, 1994). Grooves with a depth of 0.2 mm were formed as a meander to enhance the turbulence. The dialysis membrane was made of regenerated cellulose (cut off 10000, Reichelt, Heidelberg, Germany). The gas dialysis membrane was made of Teflon and had a pore size of 0.2 µm (Sartorius, Heidelberg, Germany). All connecting tubes were made of Teflon with an inner diameter of 0.5 mm (Latek, Eppelheim, Germany). The injection volume was 54 µl.

All carrier flows contained 0.2 M sodium sulphate, 0.2 M tris(hydroxymethyl)-aminomethane and 0.02 % (w/v) sodium azide. The pH of the buffers was adjusted to the optimum pH with concentrated sulphuric acid. 2.5 mM NAD was added to the carrier solution, pH 5.0, to diminish the decomposition of the NAD. A sulphuric acid solution (1 % (v/v)) was used to clean the tubes of the sample system after every analysis cycle.

The aqueous OPA solution was based on the reagent described by Roth (1971). Mercaptoethanol was replaced for thioglycolate. The complete elimination of methanol allowed gas dialysis across the hydrophobic membrane. The OPA solution consisted of 2.0 g l^{-1} o-phthaldialdehyde, 28.7 mM thioglycolic acid in 0.4 M borate buffer, pH 10.4. The flow velocity of the three carrier/reagent solutions (pH 7.15, pH 9.0 and OPA) was 0.8 ml min^{-1}.

The calibration solutions were made in culture medium containing 0.02 % (w/v) sodium azide. The concentration ranges for calibration were per liter: glucose 0.63 - 2.50 g, lactate 0.63 - 2.50 g, glutamine 112.5 - 450.0 mg, glutamate 7.5 - 30.0 mg and ammonium 18 - 72 mg.

Enzyme immobilization

All enzymes were immobilized on aminopropylsilylated controlled-pore glass (Bioran, Schott, Mainz, Germany), with a particle diameter of 150-200 μm. Glutardialdehyde was used as immobilization reagent as described by Weetall and Filbert (1974). The Schiff's bases formed were reduced with sodium borohydride for stabilizing the chemical bonding (except for glutaminase).

200 mg porous glass was activated in 1.5 % (v/v) glutardialdehyde, 0.1 M potassium phosphate buffer (KPi), pH 7.0, for 30 min under vacuum. The activated porous glass carriers were washed with de-ionized water and added to 2 ml of enzyme solution, 0.1 M KPi, pH 7.0, for 30 min under vacuum. 2 mg sodium borohydride was submitted afterwards and the suspension was kept at 4 °C overnight. Next day, the glass carriers were washed with 0.1 M KPi, pH 7.0. The immobilized amount of enzymes and the dimensions of the enzyme cartridges are shown in Table 1. The enzyme cartridges were made of Lucite.

Table 1. The amount of immobilized enzymes and the dimensions of the enzyme cartridges.

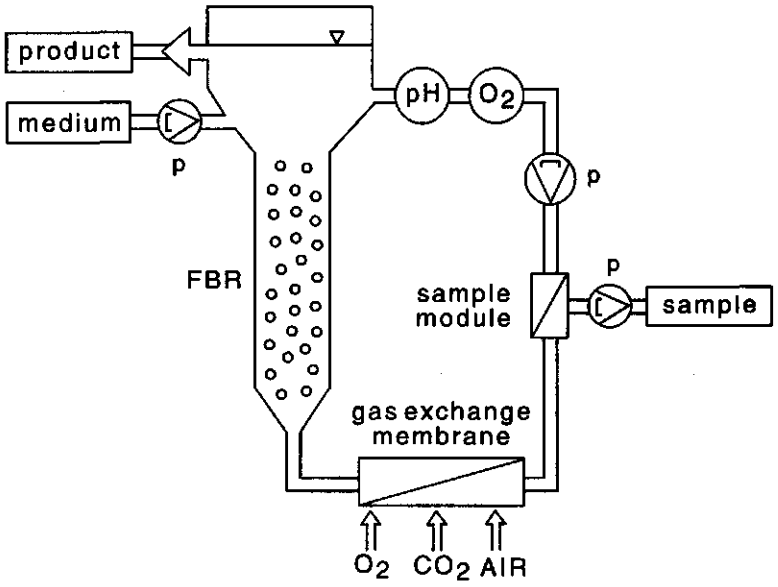
Enzymes	Units	Protein	Cartridges	
		(mg)	Length (mm)	Diameter (mm)
Glucose dehydrogenase.	250	1.14	20	2.5
Mutarotase	2300	0.88		
Lactate dehydrogenase	14200	19.70	30	2.0
Glutamate dehydrogenase	1600	37.21	30	3.0
Glutaminase	50	0.55	30	3.0

The control program (software)

The control program FIACCO (Flow-injection Analysis Control and Configuration) of the multi-channel FIA-manifold is described in detail by Spohn *et al.* (1994). The program could handle four piston pumps, six 3/2-way valves, two injection valves and two 6-way valves. In this way, maximal six different assays could be programmed independently. The activation or inactivation of the hardware components during the analysis were set via time schedules. Moreover, the flow rates of the piston pumps for pumping and refilling could be set in the time schedules. For every assay, time schedules were available for on-line analysis, off-line analysis, calibration, recalibration, pre-rinsing and after-rinsing. A typical analysis for one component started with pre-rinsing of the system to stabilise the baseline, followed by several injections of the sample or standard (analysis or calibration time schedule) and ended with rinsing of the system (after-rinsing time schedule). The number of analysis cycles after which a new calibration should be carried out, could be set in the program. The peaks measured by the detector were processed by the control program, and concentrations were calculated using the calibration curves.

The culture system

The fluidized-bed reactor (Fig. 2) consisted of a jacketed glass tube, which was inserted in an external recirculation loop. A dissolved-oxygen probe (Ingold, Steinbach, Germany), a pH probe (Ingold, Steinbach, Germany), an on-line sample system and an external oxygenator for bubble-free oxygenation were implemented in the recirculation loop. The total reactor volume was 170 ml, the maximum dilution rate was 0.44 h^{-1} (75 ml h^{-1}). The open porous microcarriers were made of borosilicate glass (Siran^R, Schott, Mainz, Germany). The glass surface was modified with gelatine by an adsorptive process (Lüllau *et al.*, 1992). The cell line was a mouse/mouse hybridoma strain producing an IgG_{2a} antibody. The medium was composed of a 3:1 mixture of DMEM and Ham's F-12 (GIBCO, Eggenstein, Germany), supplemented with the substances listed in Table 2. The final concentration of glutamine was 5 mM, of glucose 20 mM and of glutamate 25 µM.



Fig, 2. Flow diagram of the fluidized-bed reactor with external loop. FBR = fluidized-bed reactor, P = peristaltic pump.

Table 2. Supplements of the medium.

Component	Concentration	
HEPES	20.0	mM
$NaHCO_3$	2.4	g l^{-1}
BSA	0.1	g l^{-1}
Ethanolamine	0.1	mM
Na_2SeO_3	10^{-6}	mM
Fatty acids	0.61	mg l^{-1}
Insulin	0.5	mg l^{-1}
Transferrin	0.5	mg l^{-1}
Glutamine	2.0	mM
Fetal calf serum	1.0	% (v/v)

The sample system

The sample system was integrated in the recirculation loop of the reactor (Fig. 2). The cross-flow filtration module is similar to a module proposed by Ross (1986), but was further developed with regard to sterility and robustness. A microporous polypropylene tube, inner diameter 5.5 mm, mean pore size 0.2 μM (Accurel PP, Enka, Wuppertal, Germany), was fixed in a stain-less steel pipe (Fig. 3). The membrane surface was 17 cm^2. The volume of the sample system at the filtrate-side, inclusive membrane and tubes, was approximately 1.5 ml. The membrane was hydrophilized with 96 % (v/v) ethanol after steam sterilization. When necessary, the sample system was exchanged under a sterile hood.

The time delay from reactor to analysis was determined experimentally by measuring the glucose concentration with the FIA-manifold (only glucose determination) after a glucose pulse into the reactor. The time at which the glucose concentration stabilized was 30 min with a filtrate flow of 0.25 ml min^{-1} and this time interval was taken as the time delay.

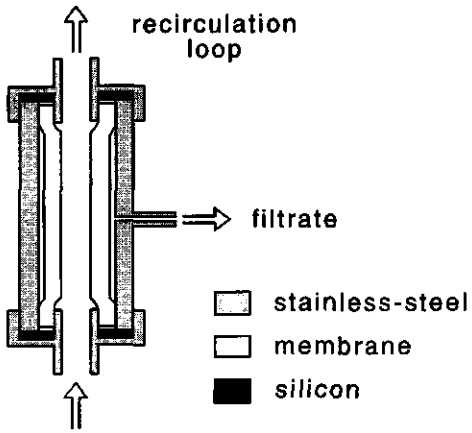


Fig. 3. Cross-flow microfiltration module for on-line sampling.

Off-line analysis

To control the FIA measurements, off-line samples were taken and analyzed. After the circulation pump was stopped, the reactor was put under a sterile hood to avoid contamination and a sample was taken from just above the microcarriers. Glucose and lactate were measured with a YSI 2000 analyser (Yellow-Springs, USA). Glutamine was measured in a combined enzyme test consisting of hydrolysis to glutamate by glutaminase and subsequent glutamate detection with glutamate dehydrogenase (Eq. 4 + 5) according to Bergmeyer *et al.* (1985a). The reverse reaction of reductive amination of α–ketoglutarate by glutamate dehydrogenase was used for the ammonium detection according to Bergmeyer *et al.* (1985b).

RESULTS AND DISCUSSION

The complexity of the culture medium required adaptation of the sample to the optimum conditions of the analytical reactions. The adaptation of the sample was carried out in the FIA-manifold via dialysis, gas dialysis and adjustment of the pH. Dialysis was performed to prevent pollution of the system and interference with the detection by proteins in the sample. The dialysis was used as an additional dilution step, to adapt the concentration range to the sensitivity of each assay. The dilution factor was set by the flow rates of the donor and acceptor solution.

To adjust the pH of sample to the pH optima of the enzymes, two carrier solutions (pH 5.0 and pH 9.0) were mixed in different ratios (Table 3). In this way, the use of more pumps could be avoided. For the measurement of the glucose and lactate concentration, the two carriers were mixed before the enzyme reactors. The immobilized glutaminase and glutamate dehydrogenase, used for the determination of the glutamine concentration, have two extremely different pH optima of pH 4.9 and 8.0, respectively. Consequently, the sample was lead over the immobilized glutaminase with the carrier solution pH 5 after which the pH was adjusted to the optimum of glutamate dehydrogenase by mixing with the carrier solution pH 9.

Table 3. pH and carrier flows for the enzymatic reactions.

Enzyme	Flow pH 5.0 (ml min^{-1})	Flow pH 9.0 (ml min^{-1})	pH	Flow pH 7.15 (ml min^{-1})
Glucose dehydrogenase	1.7	0.4	7.65	2.3
Lactate dehydrogenase	0.9	1.4	8.4	0.9
Glutaminase	0.7	-	5.0	0.7
Glutamate dehydrogenase	0.7	1.1	8.4	0.7

As cell-culture medium contains glutamate and glutamine, both had to be measured. The difference between the maximum peak height of the two measurements was used to determine the glutamine concentration. For the glutamate measurement, a blank reactor only filled with carrier material was used to compensate for the dispersion occurring in the glutaminase reactor. However, the glutamate concentration showed no significant variation during the cultures and is therefore not further mentioned.

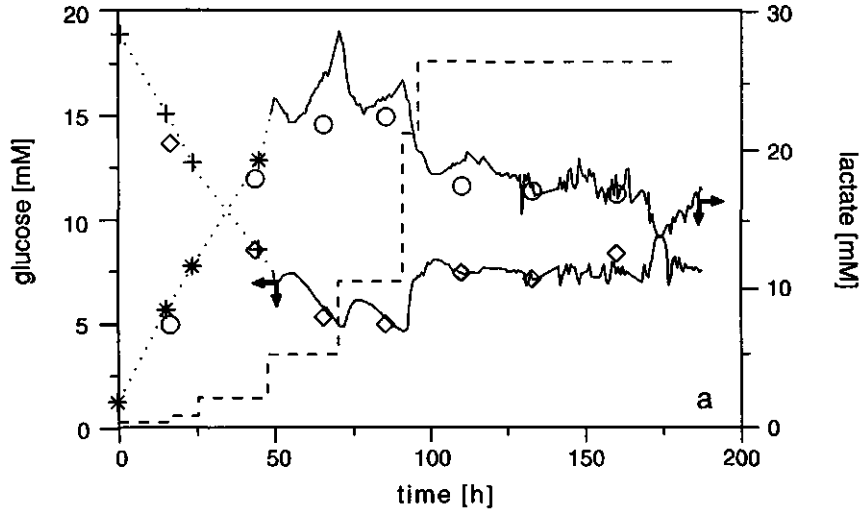
The use of OPA as reagent for the measurement of the ammonium concentration required the separation of ammonium from other amino-groups present in the sample. This was achieved by gas dialysis across a hydrophobic membrane. To enable the utilization of a hydrophobic membrane, the methanol used in the OPA solution published by Roth (1971), was eliminated. Before gas dialysis took place, the ammonium was converted to ammonia by using an acceptor solution of pH 9.0 for the dialysis (instead of pH 7.2). While the ground fluorescence of the OPA reagent was higher than the other carrier solutions, the baseline was adjusted by switching the autozero of the detector electronically before and after the ammonium determination.

So as to compensate for possible inactivation of the immobilized enzymes, the system was calibrated once a day with four concentrations for every component. The concentration of the calibration solutions were adapted to the expected concentrations during the culture and were not the limits of the determination ranges for the FIA-system. No peaks were detected measuring medium without the analytes, and were therefore not measured during the on-line measurements. One complete measurement of all components with two injections for glucose, lactate and glutamate and three injections for glutamine and ammonium took 42 min, including a final washing of the tubes of the sample system for a period of 5 min. This resulted in a sample frequency of 1.43 h^{-1}.

On-line monitoring

Two cultures were monitored on-line. The start-up of a culture was monitored for a period of 200 h The influence of the dissolved-oxygen concentration (DOC) on the metabolic rates of the cells was investigated during the second culture. This culture lasted almost 1000 h.

Fig. 4 shows the concentrations of glucose, lactate, glutamine and ammonium measured with the FIA-system and the off-line samples for the start-up period of the reactor. The feed rate was doubled once a day. The low dilution rate in the first 48 h prevented continuous sampling, so that only three measurements were made with the FIA-system during this period. The difference between the off-line and on-line values for glucose, glutamine and ammonium were not significant, whereas the differences for lactate were higher. The trend of the on-line measurements however is more consistent as the off-line data, leveling off after rising the dilution rate and increasing with higher glucose consumption.



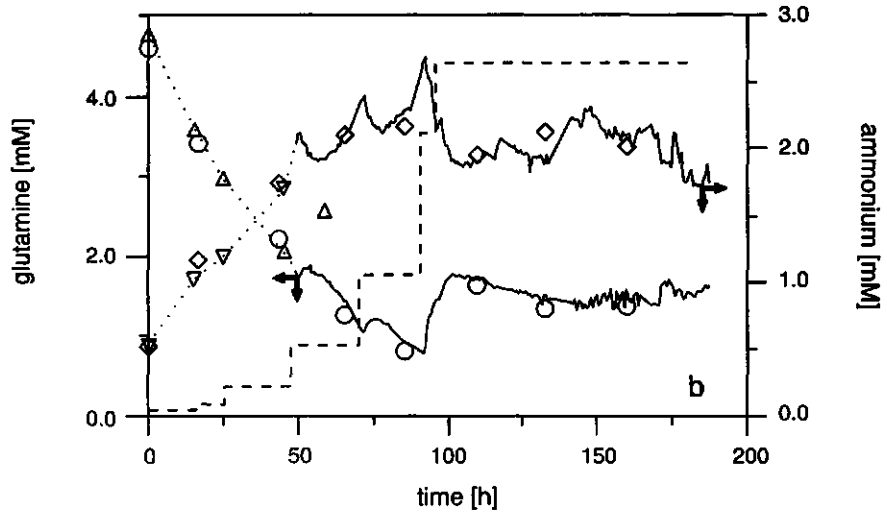


Fig. 4. Course of the concentrations of substrates and metabolites during the start-up period of the fluidized-bed reactor. The lines represent data of the multi-channel FIA system. The broken line shows the dilution rate (D_{max} = 0.44 h^{-1}). The first 48 h, the FIA-manifold measured discontinuously (dotted lines). (a) Off-line data of glucose (◇), and lactate (○), discontinuous measurements of glucose (+) and lactate (*) with FIA-manifold. (b) Off-line data of glutamine (○), and ammonium (◇), discontinuous measurements of glutamine (△) and ammonium (▽) with FIA-manifold.

The fluctuation of the measurements after about 130 h was caused by fouling of the microfiltration membrane of the sample system. This fouling induced air-bubbles in the sample stream which disturbed the measurements. Therefore, an exchange of the sample module was enforced. The time interval taken to change the sample module depended on the concentration of free cells in suspension, which were released from the fully grown microcarriers. At low concentrations of cells, the sample module could be used for 120 h. At the highest cell concentration, $1*10^6$ free cells ml^{-1}, the module had to be exchanged every 72 h.

The off-line samples outlined the time course of the concentrations incomplete, whereas the on-line data described the transition state of the concentrations very accurately as a result of the higher sample frequency. The higher density of data can be used to extract kinetic data which normally require steady-state conditions. For example, the growth rate can not be determined during a start-up period of the fluidized-bed reactor, because newly grown cells will not be detectable in suspension as long as free space is available in the microcarriers. Yet, the volumetric consumption rate will simultaneously increase with the growing population of cells which can be correlated to the alteration of the substrate concentration (S) by the mass balance over the reactor. However, the mass balance has to be extended with an exponentially rising consumption rate (r) as a consequence of the exponential growth rate (μ) of the cells. This resulted in Eq. 7, which describes the alternation of the substrate concentration after an increase in the feed rate (F):

$$\frac{d(C_S)}{dt} = \frac{F}{V}(C_{S,in} - C_{S,out}) + r_o e^{\mu t} \qquad (7)$$

whereby r_0 represents the consumption rate at the time the feed rate is raised (t = 0 h), V the total reactor volume, $C_{S,in}$ the concentration of the substrate in the feed and $C_{S,out}$ the concentration of the substrate in the reactor and outlet.

An estimation of the growth rate can be made by fitting Eq. 7 to the on-line measured substrate concentrations. Fig. 5 shows an example for the course of the glucose and glutamine concentrations after enhancing the dilution rate. The growth rate estimated using the course of the glucose concentration was for the first increase 0.021 h^{-1} and for the second increase 0.018 h^{-1}. Using the course of the glutamine concentration for the same periods resulted in estimated growth rates of 0.024 h^{-1} and 0.017 h^{-1}, respectively. The lower growth rate for the second increase can be attributed to either a lower glutamine concentration or to less free surface in the microcarriers. The maximum growth rate for this cell line in suspension is 0.05 h^{-1} (data not shown). The decline of the growth rate after 85 h, was probably caused by the increasing medium depletion.

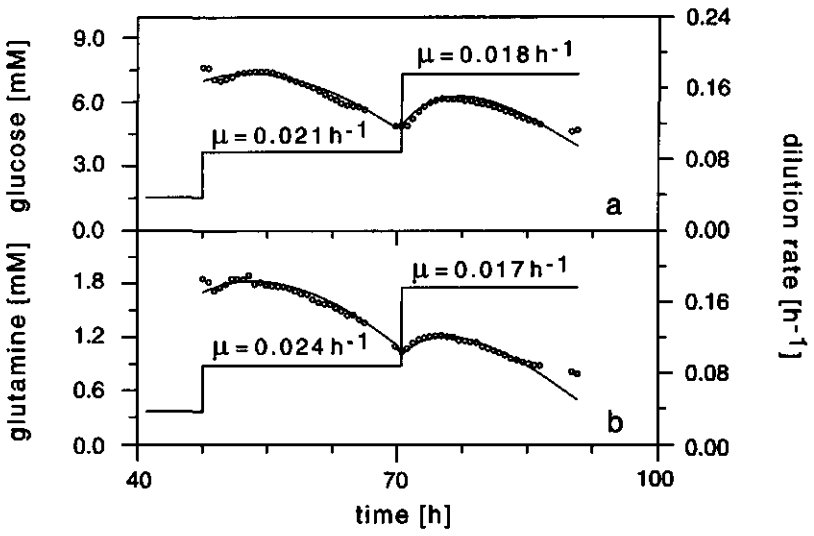


Fig. 5. Estimation of the growth rate (μ) by fitting Eq. 7 (−) to the course of the concentrations for glucose (a) and glutamine (b) (○). t = 0 h at the time the dilution rate is increased. The dilution rate (broken lines) was increased from 0.0368 h^{-1} to 0.0882 h^{-1}, and from 0.0882 h^{-1} to 0.176 h^{-1}.

Fig. 6 shows the course of the concentrations of glucose and lactate and of glutamine and ammonium, respectively, during an investigation on the influence of the dissolved-oxygen concentration (DOC) on the cell metabolism. The pattern for the start-up period of the reactor was almost identical as described above. After 225 h the amount of microcarriers and the dilution rate were diminished to lower the medium consumption. The differences between the off-line and FIA-values for glucose, glutamine and ammonium were not significant. The off-line values for lactate were higher than the values measured with the FIA-system. However, the trend of the lactate concentration is considered the same. The periodically occurring small peaks observed in the FIA values were caused by a fast refilling of the reactor with fresh medium after taking off-line samples, changing the sample system or withdrawal of protein clumps out of the reactor.

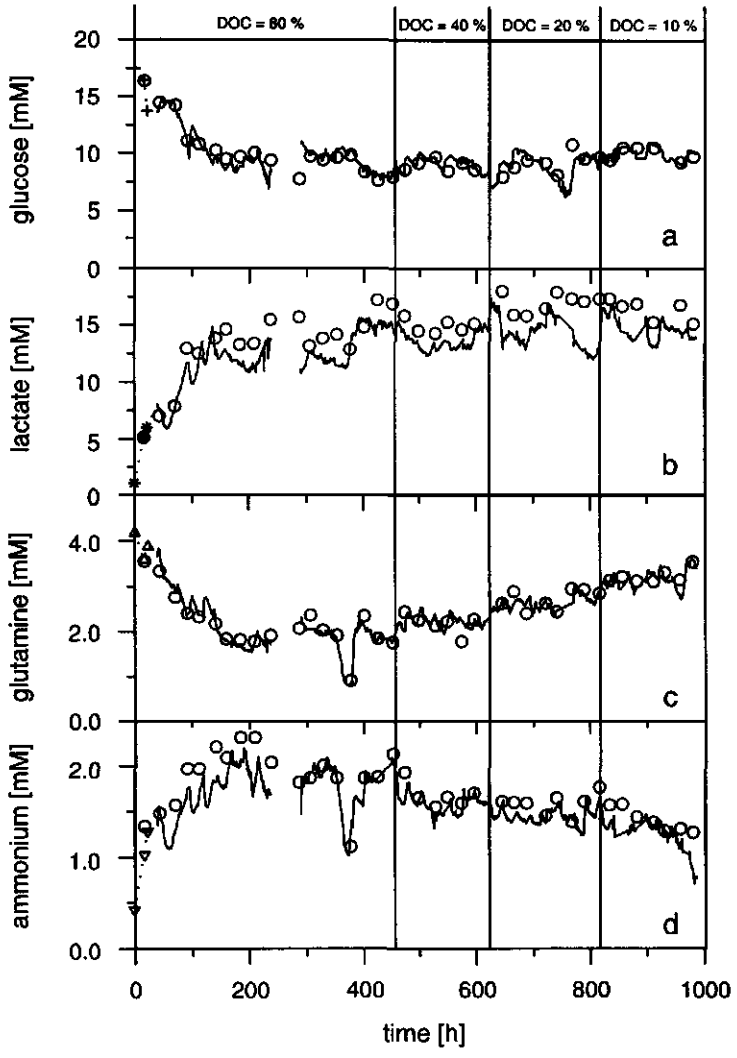


Fig. 6. Influence of DOC on the concentration of glucose (a), lactate (b), glutamine (c) and ammonium (d). Data of the multi-channel FIA-system are represented by lines, off-line data by (○). The boundaries for periods of different DOC are represented by the vertical lines. The first 48 h, the FIA-manifold measured discontinuously (dotted lines) the concentrations of glucose (+) (a), lactate (*) (b), glutamine (△) (c) and ammonium (▽) (d).

After 370 h, the glutamine concentration in the feed was lowered from 5 mM to 3 mM. The cells reacted very quickly, both the glutamine consumption rate and the ammonium production rate being reduced (Fig. 7). The concentration of lactate and glucose remain constant during the decrease of the glutamine concentration. The concentration of glutamine in the feed was changed back from 3 mM to 5 mM 20 h later. The glutamine consumption rate and ammonium production rate reached their previous level. However, this time the glucose consumption rate and the lactate production rate increased. The above described reaction of the cells shows that they can react fast to changing conditions despite of their slow growth rate and that a sufficient high analysis frequency is required to monitor animal-cell cultures.

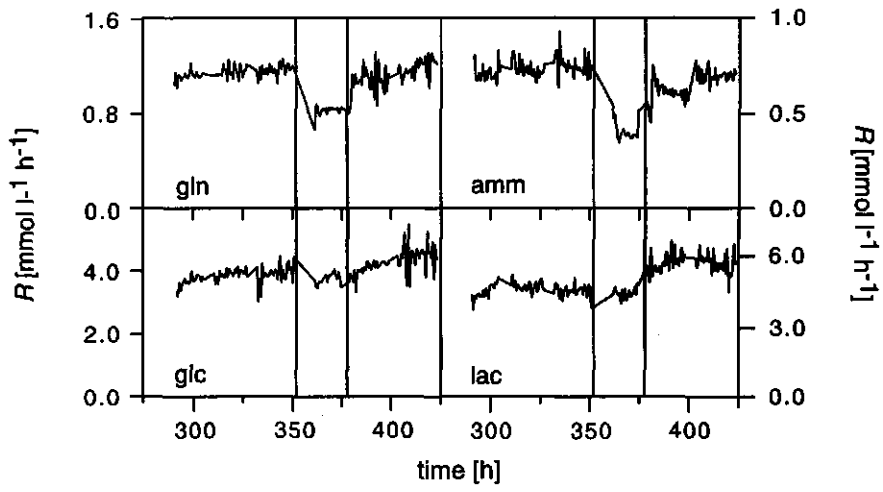


Fig. 7. Course of the metabolic rates (R) of glutamine (gln), glucose (glc), ammonium (amm) and lactate (lac) during a sudden change of the glutamine concentration from 5 mM to 3 mM (and visa versa) in the feed. The vertical lines represent the time at which the changes took place.

The influence of the DOC on the metabolic rates of the measured analytes is similarly as described by Thömmes *et al.* (1993). The glutamine consumption rate decreased with decreasing DOC, because most of the glutamine is oxidised producing ammonium (Fig. 8). The rate at which glucose is converted to lactate via glycolysis was hardly influenced by the decrease of the DOC (Fig. 8).

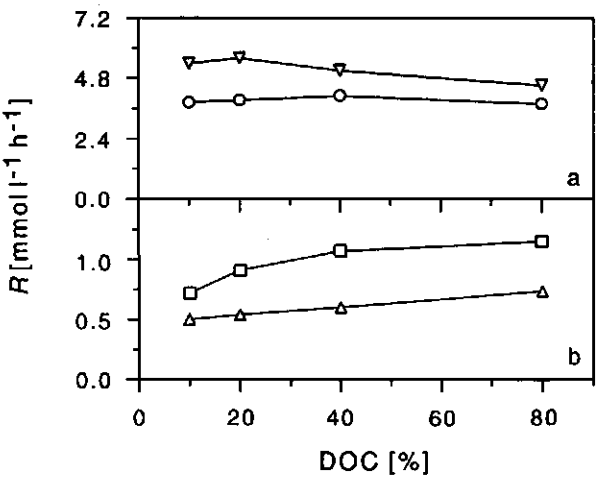


Fig. 8. The influence of the dissolved-oxygen concentration (DOC) on the metabolic rates (R) of (a) glucose (○) and lactate (▽), (b) glutamine (□) and ammonium (△).

Operational stability of the analysis device

To maintain the analysis system, the buffer solutions and the calibration solutions had to be replenished daily and weekly, respectively. No other routine labour was required. However, as is common with other on-line analysis devices, some failures occurred during the long experimental time. These failures were due to either electronic and human failures or to obstruction of the connection tubes. Electronic failures were due to attrition of the electronical position contacts of the 6-way and the

injection valves. These failures caused sporadic false measurements which are left off the figures. Human failures were: changing the sample module too late (2 times), changing the gas dialysis membrane too late (1 time) and not checking the position of the piston pumps after refilling the reagent solutions (1 time). Air bubbles appeared when the sample module was used too long. This occurred during the first culture at the end of the run (Fig. 4) and during the second culture between 240 - 290 h (Fig. 6). At the end of the second experiment, around 850 h, the baseline for the ammonium measurement was unsteady. After changing the gas dialysis membrane, the baseline was stable again. The piston pumps can be in an inactive mode after refilling the solutions, because the connection between the motor and pistons is easily disrupted. This happened during the second culture for the ammonium measurement, which was around 400 h. Around 750 h, one of the connecting tubes was blocked, causing an anomaly for glucose, lactate and glutamine. The software did not produce any failure during the course of the measurements.

Although some problems were experienced during the on-line measurements, it is shown that the analysis system is stable enough for on-line control of animal-cell cultures. The above mentioned problems can be eradicated by an automated checking of the hardware by the control software and regular exchange of sample module and gas dialysis membrane.

Operational stability of the enzyme reactors under process conditions

The calibration curves for glucose, glutamine and ammonium were straight lines and had correlation coefficients between 0.995 and 1.000. Lactate showed a square-root curve. The determinant of the curve was also 0.995 or higher. Fig. 9 shows the calibration curves of the enzymatic assays during an almost continuous use over a period of 2 months. The glutamate dehydrogenase reactor was renewed once during two months. This renewal was due to the double measurements of glutamine and glutamate. The amount of injections analyzed with one enzyme reactor were for glucose and lactate dehydrogenase 3000 injections, for glutaminase 4500 injections and for glutamate 5000 injections. The inactivation of the enzymes was corrected by calibration.

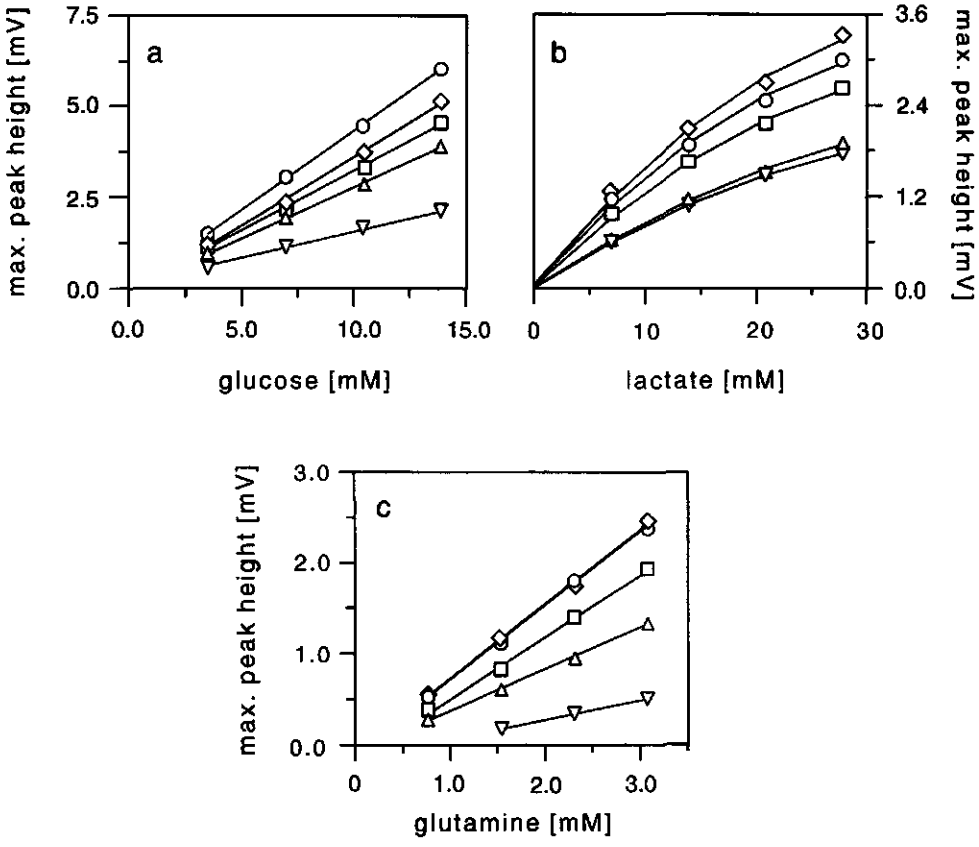


Fig. 9. Calibration curves for the enzymatic assay over a period of 55 d. Correlation coefficients for glucose (a) and glutamine (b) were 0.995 or higher. The determination coefficients for lactate (c) were 0.995 or higher. t = 0 d (○), t = 14 d (◇), t = 27 days (□), t = 41 d (△), t = 55 d (▽).

CONCLUSIONS

The time course of the concentrations of glucose, lactate, glutamine and ammonium during hybridoma cultures could be monitored up to 1000 h continuously, with one multi-channel flow-injection manifold. The combination of software and hardware used in the manifold, allowed

methods, e.g. dialysis, gas dialysis and mixing of two buffers in different ratios, to adjust the sample to the optimum conditions for enzymatic and chemical assays. This resulted in the successful integration of three enzymatic assays with immobilized enzymes and one chemical assay into one manifold. Although the hardware occasionally failed when analyzing continuously over a period of almost 2 months, the hardware and software were, in general, very stable. The immobilized enzymes were operational for 3000 - 5000 injections, whereby inactivation of the enzymes was corrected through daily calibration.

As a result of the higher analysis frequency (every 42 min), on-line monitoring represented much better the actual state of a culture compared to off-line samples. Monitoring the time course of the analytes after a shift of the glutamine concentration in the feed showed that animal-cells can adapt their metabolic rates very quickly. Such a quick response will be amplified in high cell-density cultures which makes it necessary to monitor such systems with a high analysis frequency, especially when process control is the prerequisite aim. On-line monitoring may also be a powerful tool in the determination of kinetic data, for example the growth rate, from transition experiments. Moreover, the FIA-manifold could be very suitable to monitor and control transient processes like fed-batch cultures. At the present, experimental attempts are being carried out to control fed-batch and continuous cultures of hybridoma cells using the FIA-manifold.

NOMENCLATURE

$C_{S,out}$	concentration in reactor and outlet	(mM)
$C_{S,in}$	concentration in feed	(mM)
DOC	dissolved-oxygen concentration. 100 % = at air saturation.	(%)
F	feed rate	($l\ h^{-1}$)
r_0	consumption rate at t = 0 h	($mmol\ h^{-1}\ l_{reactor}^{-1}$)
t	time	(h)
μ	growth rate	(h^{-1})
V	liquid volume of reactor, assumed to be constant	(l)

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Chapter 4

On-line Control of an Immobilized Hybridoma Culture with Multi-Channel Flow-Injection Analysis

SUMMARY

An immobilized hybridoma cell line was cultivated at controlled glucose and glutamine concentrations. On-line analysis of the substrates was carried out with a multi-channel flow-injection analysis system. The analysis system also determined on-line the lactate and ammonium concentration. The substrate concentrations were controlled using an adaptive-control strategy. This strategy consisted of the estimation of the real-time concentrations and volumetric substrate consumption rates by an Extended Kalman Filter, and a minimum variance controller, which used the estimated parameters to set the feed rates of the substrates.

The closed-loop control was used to start-up two cultures with either glucose or glutamine as control-substrate for the medium feed rate. The controller kept the concentration of the control-substrate constant by enhancing the medium feed rate simultaneously to the increasing volumetric consumption rate of the substrate. When glutamine was used as control-substrate, the glucose concentration remained relatively constant, whereas the glutamine concentration decreased during the start-up at a constant glucose concentration. This indicates that glutamine is consumed faster than glucose and will be a better control-substrate to avoid limitation during the start-up of a culture with the applied hybridoma cell line. During the colonization of the microcarriers, the yield of ammonium on glutamine decreased from 0.80 to 0.55 (mol mol^{-1}), indicating a change in the glutamine metabolism. The yield of lactate on glucose stayed constant for both experiments.

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During long-term culture of more than 800 h, the controller kept both the glucose and glutamine concentrations constant at perfusion rates between 0.50 h^{-1} and 0.15 h^{-1}. The medium, glucose and glutamine feed rate were independently controlled. Both the specific glutamine and glucose consumption rates remained constant for all perfusion rates, which was probably as a result of the constant concentrations. The specific monoclonal antibody production rate decreased with the perfusion rate decreasing from 0.40 h^{-1} to 0.20 h^{-1}. The immobilized-cell concentration decreased only at the lowest perfusion rate. Both effects could not be explained directly by the increasing ammonium and lactate concentrations nor by the decreasing amino-acid concentrations.

INTRODUCTION

Animal-cell cultures are of increasing importance in the production of monoclonal antibodies and new pharmaceuticals. Since the complex production process and the expensive medium leads to relatively high prices, higher productivity is desirable. Extending the high-cell-density production period through medium supplementation, as in fed-batch and perfusion cultures, results in higher productivity (Robinson *et al.*, 1994; Jo *et al.*, 1993; Xie and Wang, 1994b). A first step to improved medium supplementation is the adjustment of the optimal concentration of the C- and N-sources, e.g. glucose and glutamine. The optimal concentration will be based on the balance between limitation of glucose and glutamine and the production of their waste products, ammonium and lactate. A strong glucose or glutamine limitation can decrease growth and production and can induce apoptosis (Singh *et al.*, 1994). Unlimited supplies of glucose and glutamine result in high concentrations of ammonium and lactate, which inhibit the growth and/or productivity (Glacken *et al.*, 1988; Lüdemann *et al.*, 1994; Taya *et al.*, 1986). Moreover, both higher ammonium concentrations and glucose starvation affect the glycosylation patterns of CHO cell lines (Borys *et al.*, 1994; Hayter *et al.*, 1993).

Controlled supplementation of glucose and glutamine can be achieved by limiting the concentrations in the feed rate. In this way Ljunggren and Häggström (1994) kept the glutamine and glucose concentration lower than 0.085 mM and 0.93 mM, respectively. However, this kind of supplementation can only be accomplished through carefully chosen feed concentrations based on experience and is sensitive to changing metabolism. Another option is the prediction of the consumption rates using a model. However, a model will be only valid for the conditions under which the model parameters are determined. Xie and Wang (1994a) reported deviations of the predicted concentrations caused by differences between the cell strain used and parameters derived from the literature. Pelletier *et al.* (1994) also reported deviations from the model-predicted course for lactate and ammonium concentrations during a perfusion culture. The difference was probably due to an altered metabolism in comparison to the batch cultures used to determine the model parameters. Tremblay *et al.* (1993) used the carbon-dioxide production rate as a feedback control to compensate for deviations of the model, in order to accomplish an optimal glutamine supplementation. However, there were still discrepancies between the predicted and the actual concentrations.

Simultaneous control of the glutamine and glucose concentrations during a fed-batch culture was accomplished by Kurokawa *et al.* (1994), using an adaptive-control algorithm. An HPLC system determined on-line the glucose, lactate and glutamine concentrations. Controlling the glucose and glutamine concentrations at $0.2\ g\ l^{-1}$ and $0.1\ g\ l^{-1}$, respectively, increased the antibody production and cell concentration two-fold in comparison with higher glucose concentrations.

Flow-injection Analysis (FIA), introduced by Ruzicka and Hansen (1975), is now an established method to automate analytical assays. The combined advantages of FIA, i.e. automated sample conditioning and short analysis time together with selective assays gives this technique a high potential for on-line monitoring and control of biotechnological processes. In spite of this, FIA systems are not often applied for process control, which could be due to reduced reliability of the often complex analytical systems (Schügerl *et al.*, 1991). In an earlier paper (Van der Pol *et al.*, 1994), we

reported the on-line monitoring of an animal-cell culture for a period of almost 1000 h with a multi-channel FIA system. Since only incidental failures occured during the long culture, we considered this analysis system stable enough to be employed for on-line control of the glucose and glutamine concentrations during culture of immobilized hybridoma cells in a fluidized-bed reactor. An adaptive-control strategy was applied, consisting of an Extended Kalman Filter and a minimum variance controller. Both elements of the controller contained simple mass balances and only the initial consumption rates and disturbance of the measurement and process were needed to start the control. Two cultures were started at either a constant glucose or a constant glutamine concentration, in order to determine the best control-substrate for regulation of the medium feed rate during the start-up of a culture. During a third culture of more than 800 h, the glucose and glutamine concentration were controlled independently at different perfusion rates.

MATERIAL AND METHODS

Chemicals

NAD and glucose dehydrogenase (EC 1.1.1.47) from *Bacillus megaterium* were purchased from Merck (Darmstadt, FRG). Lactate dehydrogenase (EC 1.1.1.27) type XI from rabbit muscle, glutamate dehydrogenase (EC 1.4.1.3) type III from bovine liver, glutaminase (EC 3.5.1.2) grade V from *Escherichia coli* and mutarotase (EC 5.1.3.3) from porcine kidney were products of Sigma (Deisenhofen, FRG). All other chemicals were analytical grade. Buffer solutions and medium were prepared with distilled/de-ionized water.

FIA-manifold

The hardware and the control program of the FIA-manifold are described in more detail by Spohn *et al.* (1994), the analytical reactions by Van der Pol *et al.* (1994). The configuration of the FIA-manifold (Fig. 1) and buffer solutions as described by Van der Pol *et al.* (1994) were optimized to shorten the analysis times. An abstract of the characteristics of the FIA-manifold follows.

The lactate, glucose and glutamine concentrations were determined using immobilized enzymes. With all three enzymatic assays, NADH was produced by dehydrogenases, which was detected by fluorescence (excitation at 340 nm, emmision at 435 nm). The glucose concentration was determined with glucose dehydrogenase and co-immobilized mutarotase to catalyse the equilibrium of α-D-glucose/β-D-glucose. The lactate concentration was measured using lactate dehydrogenase. Glutamine was first converted to glutamate by glutaminase. The glutamate formed was further converted to α-ketoglutarate in a second enzyme reactor containing immobilized glutamate dehydrogenase. The large difference in pH optima of glutaminase (pH 4.9) and glutamate dehydrogenase (pH 8.0) forced the separate immobilization of these enzymes.

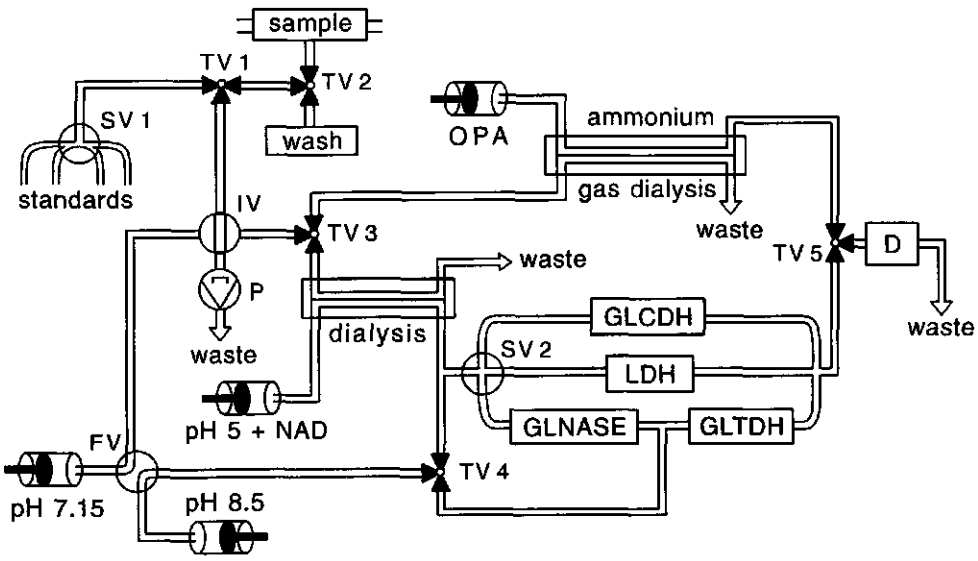

Fig. 1. Flow diagram of the flow-injection analysis manifold. IV = Injection valve; TV1-5 = 3/2-way valves; FV = 4/2-way valve; SV1-2 = 6-way valves; D = detector; GLCDH = glucose dehydrogenase and mutarotase; LDH = lactate dehydrogenase; GLNASE = glutaminase; GLTDH = glutamate dehydrogenase and OPA = o-phthaldialdehyde solution.

After injection of the sample (54 µl) into a carrier solution of pH 7.15, the sample was dialysed, to eliminate interference of high-molecular compounds and to dilute the analytes. The dialysis membrane was made of regenerated cellulose (cut off 10,000, Reichelt, Heidelberg, FRG). The acceptor solution had a pH of 5.0. After dialysis, the sample was mixed with a second buffer solution, pH 8.5, to adjust the optimal pH for the glucose and lactate determination (Table 1). For the glutamine determination, the dialysed sample was first led over the glutaminase enzyme reactor, then mixed with the buffer of pH 8.5 to adjust to the optimal pH for the glutamate dehydrogenase reactor. The buffer solutions contained 0.2 M sodium sulphate, 0.2 M tris(hydroxymethyl)-aminomethane and 0.02 % (w/v) sodium azide. The pH was adjusted with sulphuric acid.

Table 1. pH and carrier flows for the enzymatic reactions and ammonium determination.

Enzyme	Flow pH 5.0 (ml min^{-1})	Flow pH 8.5 (ml min^{-1})	pH	Flow pH 7.15 (ml min^{-1})
Glucose dehydrogenase	1.7	0.3	7.1	1.7
Lactate dehydrogenase	0.7	1.4	8.1	0.7
Glutaminase	0.6	-	5.0	0.6
Glutamate dehydrogenase	0.6	1.2	8.0	1.0

The ammonium concentration was determined using an aqueous solution of o-phthaldialdehyde (Van der Pol *et al.*, 1994). Ammonium was separated from interfering amino groups in the sample matrix by gas diffusion across a hydrophobic membrane of Teflon with a pore size of 0.2 µm (Sartorius, Heidelberg, FRG). To improve the efficiency of the gas diffusion, the ratio NH_3/NH_4 was enhanced to NH_3 by injection of the sample into the carrier solution of pH 8.5. The product of the reaction between ammonium and o-phthaldialdehyde, an iso-indol derivative, was detected by fluorescence at the same wavelengths as NADH.

The FIA-manifold was calibrated daily with four calibration solutions to correct for enzyme inactivation and drifting of the fluorescence detector. The calibration solutions were made in culture medium containing 0.02 % (w/v) sodium azide. The concentration ranges were (mM): ammonium 1.0 - 4.0, glucose 6.25 - 25.0, lactate 10.0 - 40.0 and glutamine 1.0 - 4.0. The maximum peak height of the detected reaction products was used to calculate the concentrations according to the calibration curves. One complete analysis cycle with three injections for every compound took 30 min.

Process control

The FIA-manifold was connected to a personal computer AT (IBM, Boca Raton, USA) through a SMP SYS 51 A/D data interface (Siemens, Düsseldorf, FRG). Software programs, which were developed at the Forschungszentrum Jülich GmbH, were run on the process control computer for real-time data acquisition and data evaluation (MEAS) and process control (BIOREGIE). Concurrent DOS (Digital Research, USA) was used as multi-tasking operation system.

To control the glutamine and/or glucose concentration during culture, an adaptive-control strategy (Fig. 2) was employed which could manage the non-linearity of the biological system and the time-delayed and discrete-time measurement outputs (Weuster-Botz *et al.*, 1994). A semi-continuous Extended Kalman Filter (Ray, 1981) estimated the real-time glucose ($\hat{C}_{glc}$) and glutamine ($\hat{C}_{gln}$) concentration and the volumetric consumption rates of these substrates ($\hat{R}_{glc}$, $\hat{R}_{gln}$) using the following differential equations:

$$\frac{dC}{dt} = \frac{1}{V}(F_{in}C_{in} - F_{tot}C + F_sC_s) - R_s + e_1(t) \tag{1}$$

$$\frac{dR_s}{dt} = b + e_2(t) \tag{2}$$

$$\frac{db}{dt} = 0 + e_3(t) \tag{3}$$

where V represents the reactor volume (l), F_{in} the medium feed rate (l h^{-1}), C_{in} the substrate concentration in the medium feed (mM), F_{tot} the total feed rate, F_s the additional substrate feed rate (l h^{-1}), C_s the substrate concentration in the additional feed (mM), R_s the volumetric consumption rate of substrate (mmol l^{-1} h^{-1}) and $e_i(t)$ represents the random disturbances of the process. The added state variable b is the time-dependent change of the volumetric consumption rate for the substrate. For the start-up experiments, F_s was set to 0, for culture at different perfusion rates, C_{in} was set to 0. C represents the substrate concentration (mM) in the reactor and was calculated using the following measurement equation:

$$C_m = C + f(t) \tag{4}$$

where C_m represents the measured concentration (mM) in the reactor and $f(t)$ the random error of the measurement.

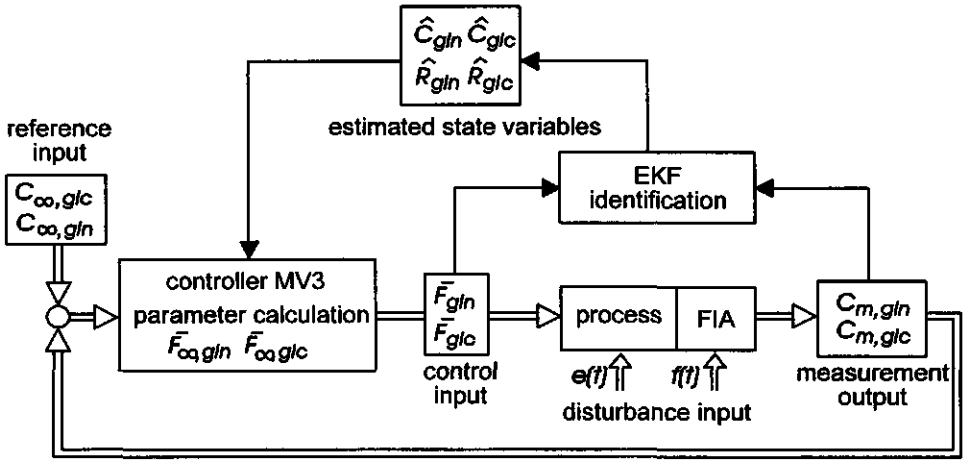

Fig. 2. Principle scheme of the adaptive-control system. Subscripts *glc* and *gln* represent glucose and glutamine, respectively. $\bar{F}$ = feed rate; F_∞ = operating flow rate of the setpoint, $\hat{C}$ = estimated concentration; C_∞ = setpoint concentration; $\hat{R}$ = estimated volumetric consumption rate, C_m = measured concentration and EFK = Extended Kalman Filter.

The estimated state variables were used to adapt the parameters of a minimum variance controller, MV3 (Isermann, 1987). The controller minimizes a function (Eq. 5), which contains the difference between setpoint ($C_{\mu,s}$) and the real-time substrate concentration predicted by the Extended Kalman Filter ($\hat{C}_s)_{t+1}$, and the difference between the operating flow rate of the setpoint ($\overline{F}_{\infty,s}$) and the real operating point ($\overline{F}_s$):

$$E = (C_{\infty,s} - \hat{C}_s)^2_{t+1} + w(\overline{F}_{\infty,s} - \overline{F}_s)^2_t \qquad (5)$$

The operating flow rate of the setpoint, was estimated from the last three measurements, using a simple mass balance for the substrate. The mass balance was solved using the estimated volumetric consumption rate of the Extended Kalman Filter. The variable w is used as a weighing factor and determines which of the two differences should be minimized faster.

Reactor

The culture system and medium are described in more detail by Van der Pol *et al.* (1994). The main characteristics are outlined briefly below. A fluidized-bed reactor (Fig. 3) with a total volume of 170 ml was used as culture system. Gas exchange was carried out by diffusion across a thin silicon tube in an external oxygenator which was integrated into the recirculation loop. The pH and dissolved-oxygen concentration were controlled at 7.2 and 80 % of air saturation, respectively, by adapting the ratio between carbon dioxide, air and oxygen in the oxygenator. The cell line was a mouse-mouse hybridoma, producing an IgG_{2a} antibody, and was immobilized on open-porous carriers (12.7 ml) made of borosilicate glass (Siran^R, Schott, Mainz, FRG). The diameter of the carrier varied between 0.56 - 0.70 mm. The glass surface of the microcarriers was modified with gelatine by an adsorptive proces (Lüllau *et al.*, 1992).

The sample module (Van der Pol *et al.*, 1994) was implemented into the recirculation loop by in-situ sterilizable valves, to enable sterile exchange of the sample module with minimal disturbance during the course of the culture. After sterilization, the polypropylene microfiltration

membrane (Accurel PP, Enka, Wuppertal, FRG) was hydrophilized with ethanol, followed by intensive rinsing with fresh medium. After filling up the sample module with fresh medium, the module was integrated into the culture system. The time delay between sampling and analysis was determined at 25 min with a sample flow rate of 0.2 ml min^{-1}.

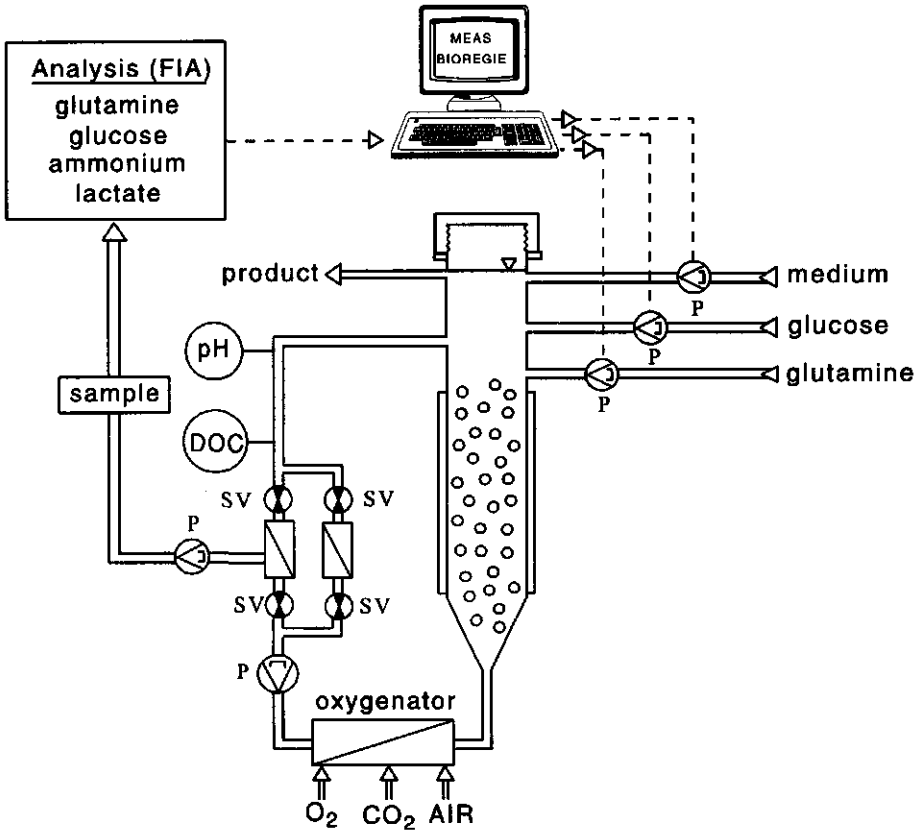

Fig. 3. Flow diagram of the fluidized-bed reactor. During the start-up experiments only one medium feed was used. SV = in-situ sterilizable valve; P = peristaltic pump, DOC = dissolved-oxygen concentration.

Two cultures were started at either a constant glutamine or a constant glucose concentration. During both start-up experiments, the cultures were fed with the same, singular feed. The feed consisted of a medium with glutamine and glucose concentrations of 5 mM and 20 mM, respectively. The medium feed rate was set by the controller with a calibrated peristaltic pump (Watson Marlow, Falmouth, GB). The setpoint for the start-up at a constant glutamine concentration was 2 mM, for the start-up at a constant glucose, the set-point was 10 mM.

During a culture at different perfusion rates, the feeds of medium, glucose and glutamine were separated. The medium contained neither glucose nor glutamine, the glucose and glutamine feed consisted of medium supplemented with either glucose (222.2 mM) or glutamine (25 mM). Two independent controllers were used to keep the concentration of glucose at 10 mM and the concentration of glutamine at 2 mM. The medium feed rate was produced by a calibrated peristaltic pump (Watson Marlow), the additional feed rates of glucose and glutamine were controlled gravimetrically with balances (Sartorius Dose system, Göttingen, FRG). The perfusion rate was kept constant by adjusting automatically the medium feed rate to the difference between the selected perfusion rate and the sum of the glutamine and glucose feed rate.

Off-line measurements

Cell counts

The concentration of viable cells in suspensions was determined using the erythrosine B exclusion method (Philips, 1973).

The concentration of immobilized cells was determined at the end of a stationary state. A sample volume of approximately 0.5 ml microcarrier was taken out of the reactor. The medium was quickly withdrawn and 2 ml of 0.1 M citrate buffer in Phoshate-Buffered-Saline (PBS), pH 7.2 was added and shaken vigourously. PBS consisted of 137 mM NaCl, 27 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4. The carriers were incubated at 37 °C for 20 min and the cells in suspension were counted with

erythrosine B. After counting was complete, the citrate buffer was withdrawn and the procedure was repeated. The carriers were washed with water and dried. The volume of the carriers was calculated using a calibration curve (not shown).

Monoclonal antibodies

The antibody concentration was determined by HPLC (2156, Pharmacia LKB, Freiburg, FRG), using a ProAnaMab column (Biolytica, Lund, Sweden). 0.5 ml of sample was injected in a 0.11 M sodiumacetate buffer, pH 5.0. The bound antibodies were eluted from the column with a 0.155 M glycine, pH 1.6 buffer. The values were corrected for the serum IgG by measuring original medium.

Amino acids

The amino-acid concentrations were determined by HPLC (Hewlett Packard, Waldbronn, BRD) using derivatisation with o-phthaldialdehyde (Hewlett Packard). The derivatised amino acids were separated on a reverse-phase column (RP-18 Hypersil ODS, Machery and Nagel, Düren, FRG). The amino acids were eluted with a 20 % to 80 % (v/v) gradient of methanol in 0.1 M sodium acetate. Peaks were detected with a fluorescence detector (excitation 230 nm, emission 455 nm).

Precipitation of proteins before analysis was carried out by adding 0.1 ml of trichloroacetic acid (36 % (w/v)) to a 0.5 ml sample. After centrifugation, 15 µl of 4.5 M NaOH was added to 300 µl of supernatant. The sample was further diluted with PBS buffer to the desired concentration.

RESULTS AND DISCUSSION

Start-up of a culture at either a constant glutamine or glucose concentration

The controller kept the glutamine concentration constant at the setpoint of 2 mM (Fig. 4) by adjusting the single feed rate. Deviations of the setpoint occured during calibration of the FIA-system, which lasted 2 h. During this period, the controller did not receive new data and could drift from the setpoint. After the calibration, the controller compensated for the drifting which caused some fluctuation in the feed rate.

During the first 100 h of the start-up of a culture at a constant glutamine concentration (2 mM), the cells did not grow. Analysis of medium samples from the reactor revealed a low concentration of residual ethanol from the hydrophilisation of the microfiltration membrane of the sample module. After the ethanol was washed out of the reactor, the cells started to grow.

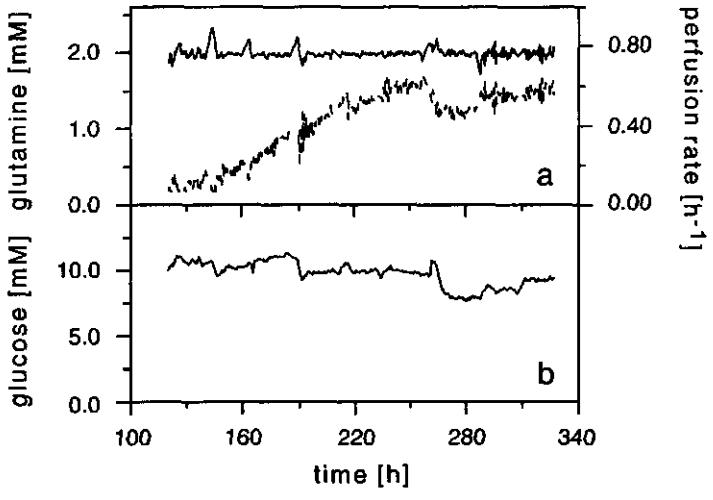

Fig 4. Course of the glutamine (a) and glucose (b) concentrations during the start-up of a culture at a constant glutamine concentration (2 mM). The broken line represents the perfusion rate.

After around 250 h, the volumetric glutamine consumption rate decreased sharply from a maximum of 1.8 mmol l^{-1} h^{-1} to 1.4 mmol l^{-1} h^{-1} followed by an increase up to 1.7 mmol l^{-1} h^{-1} (Fig. 5a). The volumetric ammonium production rate followed the same pattern as the volumetric glutamine consumption rate. The volumetric metabolic rates of glucose and lactate also decreased shortly, but returned much faster to their previous level. A consequence of the decrease of the volumetric glutamine consumption rate was a simultaneous decrease in the perfusion rate (Fig. 4a).

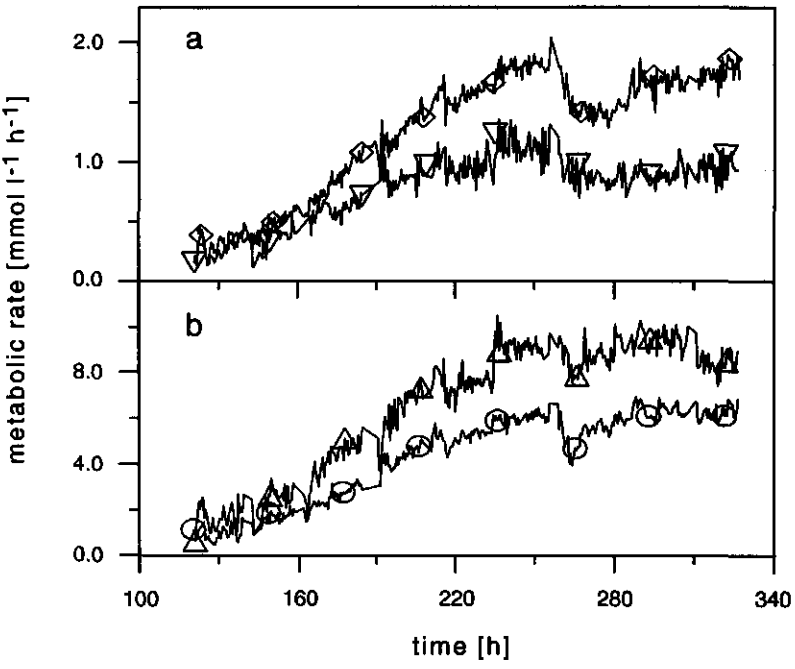

Fig 5. Course of the volumetric metabolic rates during the start-up of a culture at a constant glutamine concentration. (a) Course of the glutamine consumption rate (◇) and the ammonium production rate (▽). (b) Course of the glucose consumption rate (○) and the lactate production rate (△).

Although a decreasing perfusion rate should result in an accumulation of viable suspension cells in the reactor, their concentration decreased (Fig. 6). These viable suspension cells are considered to be newly grown cells, which can not find a free space in the microcarriers to settle (Thömmes *et al.*, 1993). The simultaneous decrease of perfusion rate and viable suspension cell concentration strongly suggest a decreasing growth rate, which probably caused the decreasing consumption rates. This effect was also found for other cell lines by Lüllau (1992). The declining growth rate could have resulted from having reached complete colonization of the microcarriers resulting in contact inhibition between cells (Hawboldt *et al.*, 1994) or diffusion limitation of a certain compound. After adaptation to the contact inhibition or limitation, the cells reached a steady growth rate.

The yield ($Y_{amm/gln}$) of ammonium on glutamine gradually decreased from approximately 0.80 to 0.55 mol mol^{-1} during culture (Fig. 7a), indicating that more glutamine is degraded through transamination reactions. Since amino-acid analysis was not carried out, it could not be determined if the nitrogen derived from glutamine was incorporated in the cell mass or was excreted as an amino acid. The yield ($Y_{lac/glc}$) of lactate on glucose stayed constant at a value of approximately 1.5 mol mol^{-1} during culture (Fig. 7b).

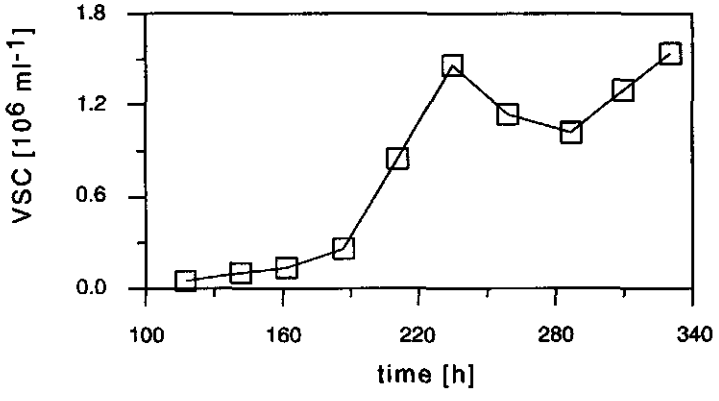

Fig 6. Course of the Vital-Suspension Cell (VSC) concentration during the start-up of a culture at a constant glutamine concentration.

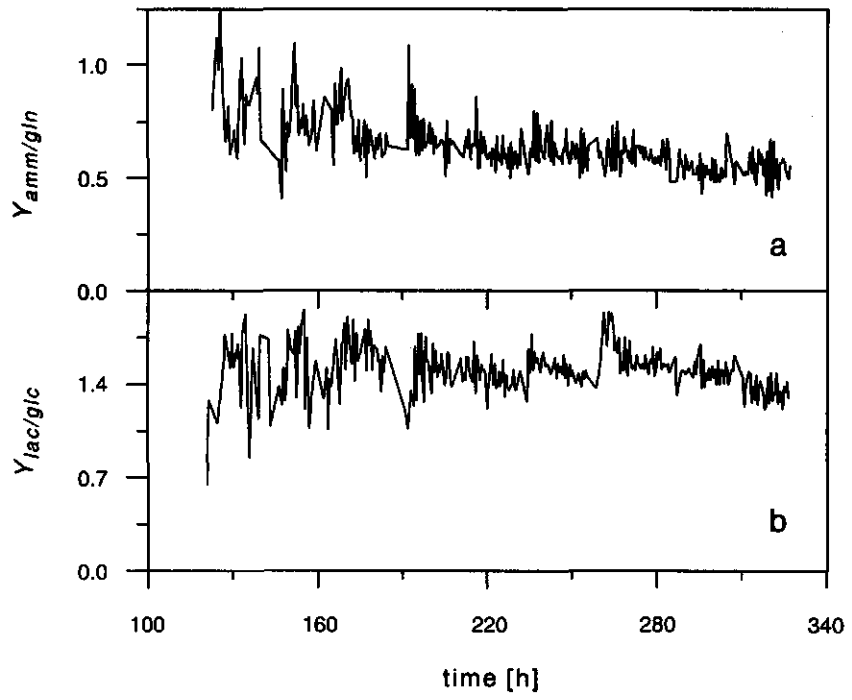

Fig. 7. (a) Course of the yield of ammonium on glutamine ($Y_{amm/gln}$) and (b) yield of lactate on glucose ($Y_{lac/glc}$) during the start-up of the culture at a constant glutamine concentration.

During the start-up of the culture at a constant glucose concentration, deviations of the setpoint (10 mM) occurred as a result of the calibration of the FIA-system (Fig. 8a). The decrease of the volumetric metabolic rates found during the start-up at a constant glutamine concentration only occurred slightly (data not shown). A decrease in the concentration of viable suspension cells did not occur. The $Y_{amm/gln}$ decreased from approximately 0.80 to 0.50 mol mol^{-1} and the $Y_{lac/glc}$ stayed constant at a level of 1.4 mol mol^{-1} (data not shown).

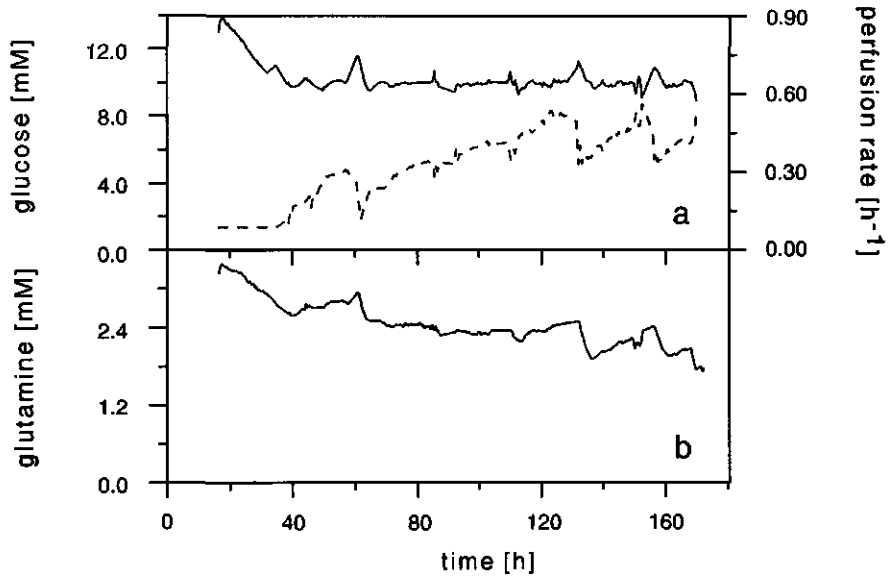

Fig. 8. Course of the glucose (a) and glutamine (b) concentrations during the start-up of a culture at a constant glucose concentration (10 mM). The broken line represents the perfusion rate.

One significant difference between the two experiments is the course of the uncontrolled substrate concentrations during the exponential growth phase. The glucose concentration stayed relatively constant during glutamine-controlled culture, indicating an equivalent increase of the volumetric consumption rates of both substrates (Fig. 4b). The glutamine concentration, however, decreased during the glucose-controlled culture, implying a faster increase in the volumetric glutamine consumption rate when compared to the volumetric glucose consumption rate (Fig. 8b). This indicates that glutamine can become limiting, when glucose is used as a control-substrate for the medium feed rate. This was also found by Rolef *et al.* (1994). Glutamine as a control-substrate for regulation of the medium feed rate will therefore be more reliable in avoiding limitations than glucose.

Continuous culture at different perfusion rates with constant glucose and glutamine concentrations

Three separate feed rates of medium supplemented either with glucose or glutamine and medium without glucose and glutamine were used to control independently both the glucose and glutamine concentration, and the perfusion rate. The perfusion rate was periodically decreased from 0.5 h^{-1} to eventually 0.15 h^{-1} after steady-state was reached (Fig. 9).

The closed-loop controllers kept the concentrations of glucose and glutamine constant with deviations of approximately 10 % of the setpoints, which were 2 mM and 10 mM, respectively. Incidental deviations up to 25 % of the setpoints occurred around 350 h.

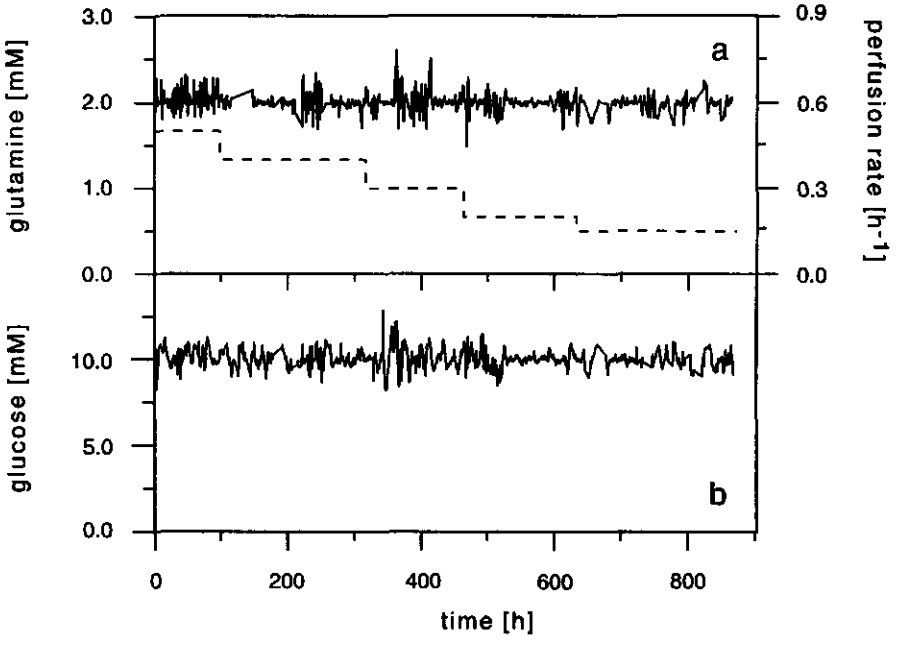

Fig. 9. Course of the glutamine (a) and glucose (b) concentrations during a culture at different perfusion rates. The glutamine and glucose concentrations were controlled at 2 mM and 10 mM, respectively. The broken line represents the perfusion rate.

The specific glutamine and glucose consumption rates were constant at values of 0.109 (± 0.012) mmol $(10^9$ cells$)^{-1}$ h^{-1} and 0.358 (± 0.030) mmol $(10^9$ cells$)^{-1}$ h^{-1} respectively, for all perfusion rates. The specific glutamine consumption rate corresponds closely with the results of Glacken *et al.* (1986), who found the following relation between glutamine concentration (C_{gln}) and specific glutamine consumption rate (q_{gln}) for immobilized Madin-Darby canine kidney cells:

$$q_{gln} = \frac{0.34 C_{gln}}{4 + C_{gln}} \text{ mmol } (10^9 \text{ cells}^{-1}) \text{ h}^{-1} \qquad (6)$$

With a glutamine concentration of 2.0 mM, Eq. 6 gives a specific glutamine consumption rate of 0.11 mmol $(10^9$ cells$)^{-1}$ h^{-1}. The constant specific glucose consumption rate was also probably the result of the constant glucose concentration (Borth *et al.*, 1990).

Two relevant effects of the declining perfusion rate were observed. A significant decrease in the immobilized-cell concentration from 5.6 (± 0.4)*10^7 cells (ml carriervolume)$^{-1}$ for the perfusion rates between 0.2 h^{-1} to 0.5 h^{-1} to 3.4*10^7 cells (ml carrier volume)$^{-1}$ at a perfusion rate of 0.15 h^{-1}. The second effect was the decrease of the specific Monoclonal AntiBody (MAB) productivity (Table 2) for a decreasing perfusion rate from 0.4 h^{-1} to 0.2 h^{-1}, although the immobilized-cell concentration was constant for these perfusion rates. The constant concentrations and specific consumption rates of glucose and glutamine for all perfusion rates excluded these substrates as a cause for the observed changes.

Other influential metabolites could be lactate and ammonium. However, the lowest specific MAB production rate was reached at a perfusion rate of 0.2 h^{-1}, whereas the highest concentrations of both metabolites were reached at a dilution rate of 0.15 h^{-1} (Table 2). Furthermore, ammonium is reported to have no influence on the specific production rate of different hybridoma cell lines (McQueen and Bailey, 1990; Newland *et al.*, 1994; Ozturk *et al.*, 1992). On the other hand, a lactate concentration of 22 mM resulted in a decrease of 30 % in the specific MAB production of a hybridoma cell line (Glacken *et al.*, 1988).

Since the immobilized-cell concentration was higher at a perfusion rate of 0.2 h^{-1}, diffusional limitation could have enhanced the concentration of lactate inside the microcarriers above the level at a perfusion rate of 0.15 h^{-1}. Therefore an estimate of the lactate concentration inside the microcarriers at the perfusion rate of 0.2 h^{-1} was made. The applied equations used to model the transport out of the microcarriers are shown in the appendix. The diffusion coefficient of lactate was assumed to be equal to the diffusion coefficient of propionic acid ($1.0*10^{-9}$ m^2 s^{-1}). The cell-diameter was estimated at $10*10^{-6}$ m and the bulk concentrations were taken from Table 2. The lactate concentrations in the center of the microcarriers were estimated at 17.0 mM and 17.5 mM for the perfusion rates of 0.2 h^{-1} and 0.15 h^{-1}, respectively. Since the specific MAB productivity was higher at the lowest perfusion rate, lactate does not seem the most influential factor. The lactate and ammonium concentrations also seem to be too low to cause the significant decrease in the immobilized-cell concentration.

Table 2. Steady-state concentrations of ammonium and lactate and the specific MAB productivity at different perfusion rates. The glucose and glutamine concentrations were constant for all perfusion rates.

Perfusion rate h^{-1}	Ammonium mM	Lactate mM	Specific MAB productivity mmol 10^9 cells^{-1} h^{-1}
0.15	2.25	16.95	0.85
0.20	1.81	15.94	0.65
0.30	1.43	11.53	0.79
0.40	1.06	8.62	1.01
0.50	0.89	6.92	0.90

Additional supply of amino acids is known to increase the cell density and longevity, however it does not influence the specific MAB production of hybridoma cell lines (Duval *et al.*, 1991; Hiller *et al.*, 1993). To investigate if one or more amino acids could be responsible for the decrease of the immobilized-cell concentration at the lowest dilution rate, the concentrations of several amino acids were determined (Table 3). A decrease of more than 50 % of the original medium concentrations was found for methionine, valine, isoleucine and leucine. The decrease in the immobilized-cell concentration could be due to the decrease of valine and isoleucine concentrations, as these have been found to have a positive effect on the viable cell number (Hiller *et al.*, 1993). In contrast, Duval *et al.* (1991) only found an increase following addition of a large number of amino acids and no influence on the cell density after the simultaneous addition of only isoleucine, leucine and valine. This first analysis shows that a further investigation of factors that result in limitation of the immobilized-cell concentration or specific MAB production will be necessary.

Table 3: Steady-state concentrations of amino acids at different perfusion rates. The glucose and glutamine concentrations were constant at all perfusion rates.

Amino acid	Medium	Perfusion rate (h^{-1})				
	(mM)	0.5	0.4	0.3	0.2	0.15
Histidine	0.17	0.15	0.15	0.13	0.11	0.10
Glycine	0.40	0.37	0.27	0.22	0.20	0.23
Threonine	0.66	0.53	0.52	0.49	0.43	0.42
Arginine	0.59	0.44	0.45	0.42	0.36	0.35
Tyrosine	0.32	0.25	0.25	0.23	0.20	0.20
Methionine	0.17	0.13	0.12	0.11	0.08	0.08
Valine	0.68	0.49	0.47	0.41	0.34	0.32
Phenylalanine	0.32	0.25	0.25	0.23	0.19	0.19
Isoleucine	0.66	0.43	0.40	0.36	0.29	0.28
Leucine	0.66	0.42	0.38	0.33	0.24	0.23
Lysine	0.74	0.58	0.57	0.50	0.47	0.41

CONCLUSIONS

The multi-channel FIA-system proved to be reliable in the control of the glucose and glutamine concentrations during animal-cell cultures of longer than 800 h. Combining the analysis system with an adaptive-control strategy enabled substrate controlled start-up of perfusion cultures. Moreover, the independent control of glucose and glutamine concentrations and perfusion rate was possible. This opens ways to investigate independently limiting factors for production and to optimize medium supplementation of perfusion and fed-batch cultures. Currently, experimental attempts are being made to control and optimize medium supplementation of fed-batch cultures.

The start-up of the fluidized-bed reactor at constant glucose or glutamine concentrations showed that glutamine as a control-substrate for regulation of the medium feed rate will be more reliable than glucose in avoiding limitations. During the colonization of the microcarriers, the $Y_{amm/gln}$ decreased, indicating a change in the glutamine metabolism.

A decreasing perfusion rate had a negative influence on the immobilized-cell concentration and specific MAB production rate. In contrast, the specific consumption rates of glucose and glutamine were constant, probably as a result of the constant glucose and glutamine concentrations. The increasing ammonium and lactate concentrations did not seem to be the most influential factors prodding these effects. Further investigations will be carried out to identify factors affecting the specific productivity and immobilized-cell concentration.

APPENDIX

Model of mass transfer out of a microcarrier

The model and the assumptions have been presented in detail by Born *et al.* (1995) at the DECHEMA-Jahrestagungen, Wiesbaden, Germany.
The mass-transfer coefficient, k_l (m s^{-1}), was calculated using the following equation (Damronglerd *et al.*, 1975):

$$Sh = \frac{k_l d_c}{D_l} = 0.763 \varepsilon_{fbr}^{-1.2} Re^{0.556} Sc^{0.333} \quad (7)$$

where Sh represents the Sherwood number, d_c the average microcarrier diameter (0.63 10^{-3} m), D_l the diffusion coefficient (m^2 s^{-1}), ε_{fbr} the liquid hold-up in a fluidized bed (0.72) and the Schmidt number (Sc) was calculated according the following equation:

$$Sc = \frac{\nu}{D_l} \quad (8)$$

where υ represents the kinematic viscosity (7.6 10^{-7} m^2 s^{-1}).

By Eq. 7, Re represents the Reynolds number and was calculated using the following equation:

$$Re = \frac{v_l d_c}{\nu} \quad (9)$$

where v_l represents the superficial liquid velocity in the fluidized bed (0.015 m s^{-1}).

The concentration at the surface, C_{surf} (mol m^{-3}) of the microcarrier was calculated using the following equation:

$$qX_c = k_l a(C_b - C_{surf}) \quad (10)$$

where q represents the specific metabolic rate (mol m^{-3} s^{-1}), X_c the amount of cells per microcarrier (1.67×10^4 $(carrier)^{-1}$), a the surface area of one microcarrier ($1.25 * 10^{-6}$ m^2) and C_b represents the concentration in the bulk liquid phase (mol m^{-3}).

The concentration in the center of the microcarrier, C_c (mol m^{-3}), was estimated using the following equation:

$$C_c = C_s - \left(\frac{R_c}{6D_{l,eff}}\right) r_c^2 \quad (11)$$

where R_c represents the volumetric consumption rate of one microcarrier (mol m^{-3} s^{-1}) and r_c represents the average radius of one microcarrier (0.32×10^{-3} m). An effective diffusion coefficient, $D_{l,eff}$ (m^2 s^{-1}) was estimated to compensate for the volume occupied by the solid material of the microcarrier and the cells:

$$D_{l,eff} = D_l\left(1 - \varepsilon_c - \frac{V_{cel} X_c}{V_c}\right) \quad (12)$$

where ε_c represents the solid phase fraction of microcarrier (0.51), V_{cel} the volume of one cell (1.77×10^{-15} m^3) and V_c represents the volume of one carrier (1.31×10^{-10} m^3)

NOMENCLATURE

a	average surface area of one microcarrier	(m^2)
C	substrate concentration	(mM)
C_b	concentration in the bulk liquid phase	(mol m^{-3})
C_c	concentration in the center of the microcarrier	(mol m^{-3})
C_{gln}	glutamine concentration	(mM)
C_{in}	concentration of a substrate in medium feed	(mM)
C_m	measured substrate concentration	(mM)
$\hat{C}_s$	estimated substrate concentration at discrete time t+1	(mM)
C_s	concentration of a substrate in separated feeds	(mM)
C_{surf}	concentration at the surface of the microcarrier	(mol m^{-3})
$C_{\infty,s}$	set point of a substrate concentration	(mM)
D_l	diffusion coefficient	(m^2 s^{-1})
$D_{l,eff}$	effective diffusion coefficient	(m^2 s^{-1})
$e_i(t)$	random disturbances of the process	(-)
$f(t)$	random error of measurement	(-)
F_{in}	medium feed rate	(l h^{-1})
F_s	separate feed rate of substrate	(l h^{-1})
$\bar{F}_s$	real operating feed rate at discrete time t	(l h^{-1})
F_{tot}	total feed rate	(l h^{-1})
$\bar{F}_{\infty,s}$	operating feed rate of the set point	(l h^{-1})
k_l	mass transfer coefficient	(m s^{-1})
q	specific metabolic rate	(mol m^{-3} s^{-1})
q_{gln}	specific glutamine consumption rate	(mmol 10^9 cells^{-1} h^{-1})
R_c	volumetric consumption rate of one microcarrier	(mol m^{-3} s^{-1})
r_c	average radius of microcarriers	(m)
Re	Reynolds number	(-)
R_s	volumetric consumption rate of substrate	(mmol l^{-1} h^{-1})
Sc	Schmidt number	(-)
Sh	Sherwood number	(-)
V_c	average volume of one microcarrier	(m^3)
V_{cel}	volume of one cell	(m^3)
w	weighing factor	(-)

X_c	amount of cells per microcarrier	(cells carrier^{-1})
$Y_{amm/gln}$	yield of ammonium on glutamine	(mol mol^{-1})
$Y_{lac/glc}$	yield of lactate on glucose	(mol mol^{-1})
ε_c	solid phase fraction of one microcarrier	(0.51)
ε_{fbr}	liquid hold-up in a fluidized bed	(0.72)
υ	kinematic viscosity of water	(m^2 s^{-1})
v_l	superficial velocity in the fluidized bed	(m s^{-1})

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Chapter 5

On-line Immunoanalysis of Monoclonal Antibodies during a Continuous Culture of Hybridoma Cells

SUMMARY

The monoclonal-antibody production of an immobilized hybridoma cell line cultivated in a fluidized-bed reactor was monitored on-line for nearly 900 h. The monoclonal antibody concentration was determined by an immuno affinity-chromatography method (ABICAP). Antibodies directed against the product, e.g. IgG, were immobilized on a micro-porous gel and packed in small columns. After all IgG present in the sample was bound to the immobilized antibodies, unbound proteins were removed by rinsing the column. Elution of the bound antibodies was followed and the antibodies were determined by fluorescence. The analytical procedure was automated with a robotic device to enable on-line measurements. The correlation between the on-line determined data and antibody concentrations measured by HPLC was linear.

A sampling system was constructed, which was based on a pneumatically actuated in-line membrane valve integrated into the circulation loop of the reactor. Separation of the cells from the sample stream was achieved by a depth filter made of glass-fibre, situated outside the reactor. Rapid obstruction of the filter by cells or cell debris and contamination of the sample system was avoided by intermittent rinsing of the sample system with a chemical solution. The intermittent rinsing of the filter, which had a surface of 4.8 cm^2, resulted in an operational capacity of up to 40 samples (1.0 l total sample volume). Both the sampling system and the analytical device functioned without failure during this long-term culture.

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The culture temperature was varied between 34 °C and 40 °C. Raising the temperature from 34 °C up to 37 °C resulted in a simultaneous increase of growth and specific antibody production rate. Specific metabolic rates of glucose, lactate, glutamine and ammonium stayed constant in this temperature range. A further enhancement of temperature up to 40 °C had a negative effect on the growth rate, whereas the specific monoclonal antibody production rate showed a small increase. The other specific metabolic rates also increased in the temperature range between 38 °C to 40 °C.

INTRODUCTION

Glycoproteins and antibodies are often produced using short-term batch cultures of animal cells. Batch production processes have the disadvantage that the period of high cell concentrations coupled to a high productivity is used inefficiently. A prolonged use of high cell density for production, e.g. by fed-batch or continuous cultures, could increase the efficiency. During long-term production processes however, genetic instability of the cell line can have a negative effect on the product quantity (Jo *et al.*, 1993) and/or quality, e.g. glycosylation patterns (Robinson *et al.*, 1994), when no selection pressure exists (Werner *et al.*, 1992). On-line monitoring of the product using a highly selective assay will create the possibility of avoiding inefficient production periods by detecting alterations in product quality and quantity. Moreover, on-line monitoring will be useful for validation purposes.

To measure high-molecular products of animal-cell cultures, antibodies are normally used in standard methods as the Enzyme-Linked Immuno-Sorbent Assay (ELISA). ELISA can be adapted for on-line monitoring by combining immobilized antibodies and Flow-Injection Analysis (FIA). With this combination, it is not necessary to reach a binding equilibrium between antibody and antigen, because of the highly reproducible reaction times of FIA (Mattiasson *et al.*, 1989). This feature shortens the assay time considerably. A disadvantage of the automated

method is the inactivation of the immobilized antibodies during dissociation of the bound antigen from the immobilized antibodies (Nilsson *et al.*, 1993). Gebbert *et al.* (1994) compensated for the effect of inactivation by regular calibration during the on-line determination of IgG with a combination of a sandwich-ELISA method and FIA.

Another on-line assay applied, is the competitive method, which is based on competition for binding places of the immobilized antibodies between previously mixed labelled antigen and native antigen of the sample. Inactivation of the bound antibodies will influence the amplitude of the response of this method but not the ratio between labelled antigen and native antigen (Mattiasson and Hakanson, 1992). The inactivation can be corrected by intermittent calibration with only labelled antigen. The competitive method was used to measure products as proinsulin (Birnbaum *et al.*, 1986), pullulanase (Degelau *et al.*, 1992), IgG (Stöcklein and Schmid, 1990) and α-amylase (Nilsson *et al.*, 1993). A disadvantage of competitive assays is that labelled antigen must be available and that the labelled antigen can be instable (Gebbert *et al.*, 1994).

The changing capacitance of a surface with immobilized antibodies was used to determine on-line the IgG concentration (Gebbert *et al.*, 1994). This direct measurement has the advantage that no additional reactions are necessary. However, regular calibration is still required to compensate for the inactivation of immobilized antibodies.

A different approach is the measurement of IgG through the formation of immuno precipitates between antibody and antigen (Worsfold *et al.*, 1985). Freitag *et al.* (1991) adapted this method for monitoring on-line the concentration of pullulanase and anti-thrombin III. The sensitivity of this method is not as high as the automated ELISA, but is sufficient to measure product concentrations during cultures.

A commercially available method, ABICAP (Antibody Immuno Column for Analytical Processes), was used to determine on-line the Monoclonal Antibody (MAB) concentration of a hybridoma culture. The hybridoma cells were immobilized on microporous carriers and cultured in a fluidized-bed reactor. On-line measurements were achieved by automating the analytical method with a robotic device. A new sample system was developed to enable the on-line measurements. This sample system

consisted of a pneumatically actuated in-line valve in combination with a depth filter situated outside the reactor.

During a culture period of approximately 900 h, steady-state values were determined in the temperature range of 34.5 °C to 40 °C. The steady-state values of the specific MAB production, growth rate and metabolic rates of the immobilized cells were compared with data from batch cultures with suspension cells (Barnabé and Butler, 1994, Bloemkolk *et al.*, 1992, Sureshkumar and Mutharasan, 1990).

MATERIAL AND METHODS

The culture system

The cells were cultivated in a fluidized-bed reactor with a total volume of 350 ml (Fig. 1). The dissolved-oxygen concentration and pH were computer controlled at 80 % of air saturation and 7.2 respectively, by adjusting the gas flow of air, oxygen and carbon dioxide with mass-flow valves (Brooks, Veenendaal, NL). The control program (MEAS) was developed in Forschungszentrum Jülich GmbH. Gas exchange took place by diffusion across a silicon tube integrated in the circulation loop. The temperature of the reactor was measured with a Pt-100 probe (Lauda, Lauda-Königshöfen, FRG), placed just above the microcarrier bed. The Pt-100 probe was connected with the controller of the waterbath (Lauda), which was set to the selected temperature.

The cell strain was a gift from Merck (Darmstad, FRG). The mouse/mouse hybridoma cell line produced an IgG_{2a} antibody (MAB 425, Murthy *et al.*, 1987) directed against the receptor for the human epidermal growth factor. The cells were negative in periodic mycoplasma tests (Boehringer, Mannheim, FRG). The cells were immobilized on porous glass carriers (Siran^R, Schott, Mainz, FRG) made of borosilicate glass. The surface of the microcarriers was modified with gelatine by an adsorptive process (Lüllau *et al.*, 1992). The total carrier volume was 85 ml. The medium as described by Van der Pol *et al.* (1994) was supplemented with 1 % (v/v) heat inactivated Fetal Calf Serum (Gibco, Eggenstein, FRG).

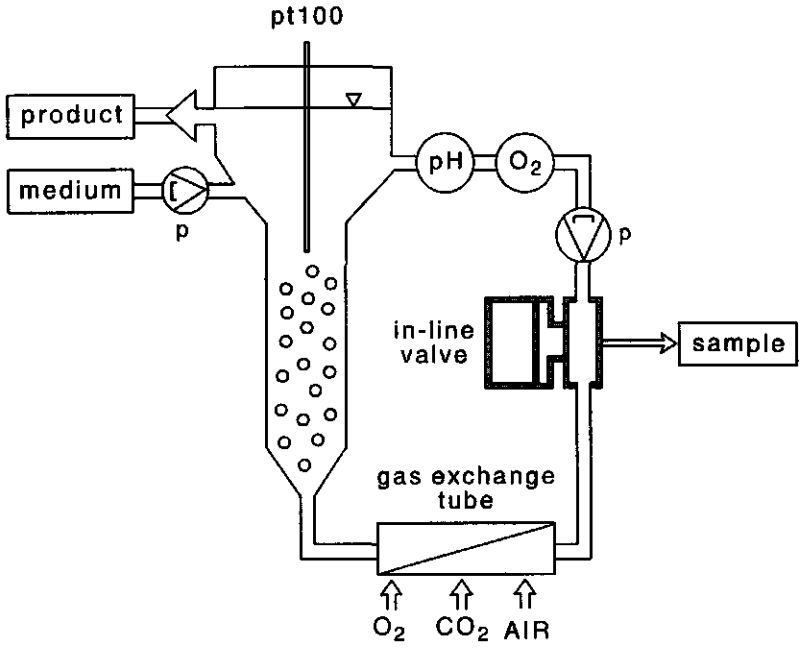


Fig. 1. Flow diagram of fluidized-bed reactor. Pt 100 = temperature probe, p = peristaltic pump.

Sample system

The sample system (Fig. 2) consisted of a stainless-steel pneumatic valve (Schico, München, FRG) integrated into the circulation loop of the fluidized-bed reactor, and a cell-separation unit placed outside the reactor. Cells were separated from the sample in a filtration unit made of Teflon (TPB 025, Amafilter, Langenhagen, FRG) which contained a depth filter made of borosilicate glass (SM 13430, Sartorius, Göttingen, FRG). The depth filter had no defined pore size and is normally used as prefilter. The filter surface was 4.8 cm^2. The filtration unit and connecting tubes were rinsed with a sterilisation and cleaning solution before and after each sampling. The solution consisted of 0.5 M NaOH, pH 13.5, 5 mM EDTA and 0.2 M $K_2S_2O_8$. An electrically-driven tube-squeeze valve (Schramme, Markdorf, FRG) blocked the tube of the solution during the sampling. The

sample was withdrawn by a peristaltic pump (U101, Watson Marlow, Falmouth, GB). The sample hole of the pneumatic, in-line valve was sealed by a membrane made of Ethylene-Propylene-Diene-Monomere (EPDM). All connecting tubes were made of Teflon with an inner diameter of 0.8 mm. The volume of the tubes and filtration unit was approximately 4 ml. The hardware components were controlled by the process computer.

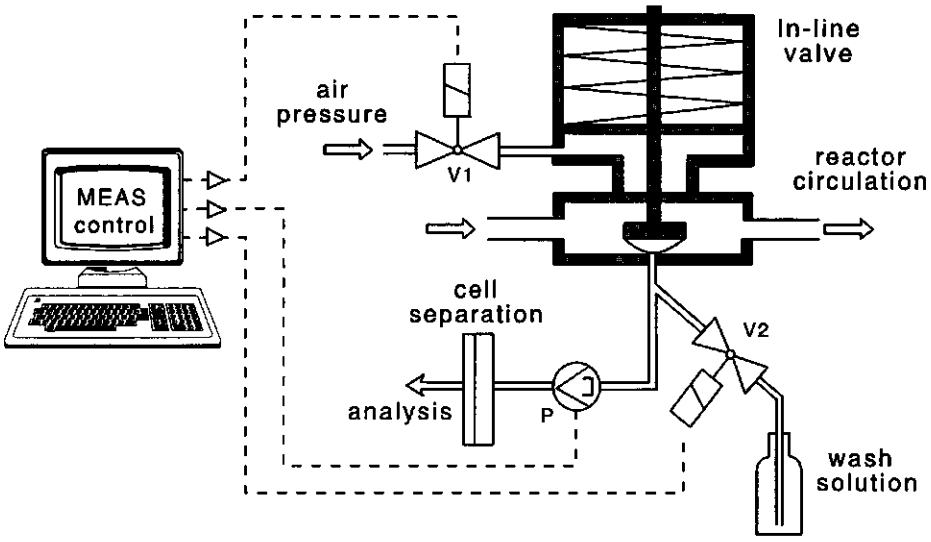


Fig. 2. The sample system. The in-line valve was integrated into the circulation loop of the fluidized-bed reactor (Fig. 1). The sampling procedure started by rinsing the sample system with the wash solution. After closing the tube-squeeze valve V2, the electrically driven valve V1 was activated so as to open the sample hole. Then peristaltic pump P withdrew medium out of the reactor. After the sample system was rinsed with medium and a sample was taken for analysis, the sample hole was closed by inactivating of valve V1. The sample procedure was ended by rinsing of the sample system with the wash solution.

On-line determination of the monoclonal antibody concentration

The on-line measurements were made with the ABICAP system (Abion, Jülich, FRG) (Fig. 3). Antibodies directed against the produced monoclonal antibodies were immobilized on a microporous gel (not specified by the producer). The gel matrix was packed into small polystyrol columns, which contain a porous filter at the bottom. A second filter was positioned at the top. Each column was used only once to avoid any influence of inactivated immobilized antibodies on the reproducibility of the assay. The small diffusional pathways in the gel, in combination with the excess of binding sites for the analyte, resulted in an endpoint assay (Hartmann *et al.*, 1993). The detection range was 5-500 mg l^{-1}.

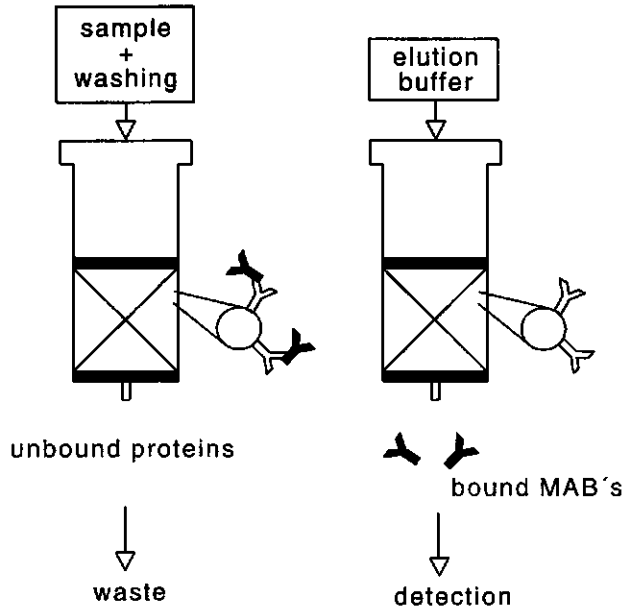


Fig. 3. The ABICAP method. After the sample is brought onto the ABICAP-column, unbound proteins are washed off the column with three washing steps. The bound MAB´s are eluted from the column and transported to the fluorescence detector (see text).

The measurements were automated with an ASPEC (Automatic Sample Preparation with Extraction Columns) automate (Gilson, Langefeld, FRG) (Fig. 4). The sample was pumped through a small box, which was closed on the uppermost side by a septum. After the complete sample system was rinsed with 25 ml of sample solution, a needle on a robotic arm passed through the septum and 500 µl of sample was withdrawn using a syringe (401, Gilson). After the sample was introduced on and passed over an ABICAP-column by gravitation, unbound proteins were removed from the column by rinsing three times with a wash solution. The wash solution consisted of 50 mM tris(hydroxymethyl)-aminomethane, pH 8.0 and 150 mM NaCl. The wash cycles were followed by elution of the bound monoclonal antibodies off the column with a buffer, which consisted of 100 mM glycine, pH 2.7 and 0.15 M NaCl. The eluent was collected in a test tube, drawn up through the needle on the robotic arm and transported to an injection valve (Rheodyne, Cotrati, USA). After the injection coil was filled, the eluent was injected into a carrier flow and transported to a fluorescence detector (RF-551, Shimadzu, Duisburg, FRG). Elution buffer was used as carrier solution. The carrier flow (0.4 ml min^{-1}) was accomplished by a low-pulse piston pump (Dosimat E665, Methrom, Filderstadt, FRG). The excitation and emission wavelength of the fluorescence detector were set to 280 nm and 340 nm, respectively.

One complete measurement consisted of measuring a sample, original medium (blank) and 0.33 µg ml^{-1} BSA dissolved in the wash solution. The BSA measurement was used to correct for small changes of the analytical device, for example drifting of the fluorescence detector, during the long-term culture.

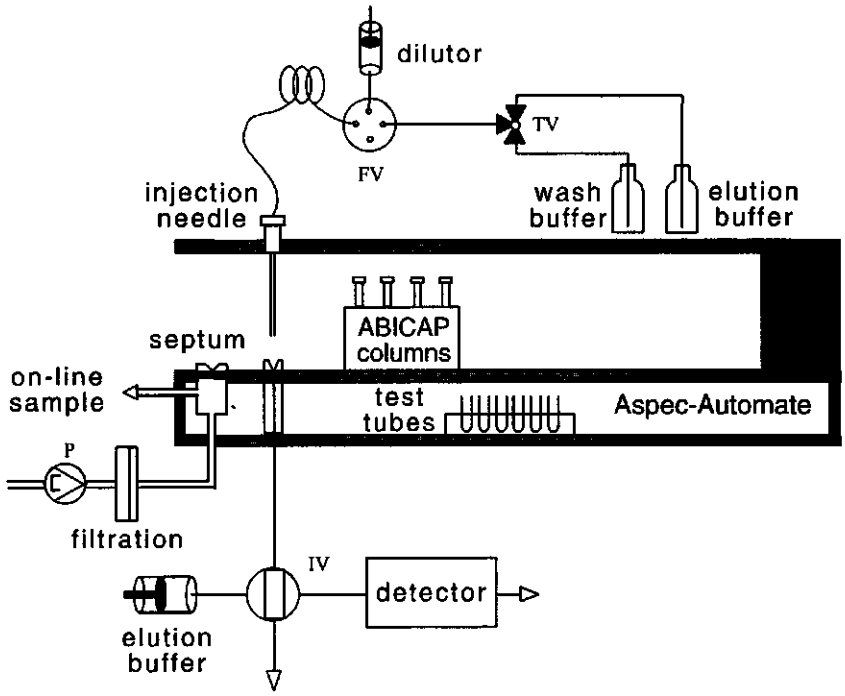


Fig. 4. The automation of the ABICAP method with an ASPEC automate. The injection needle can be moved vertically and is placed on a robotic arm, which moves freely in a horizontal plain. The measurement starts with the withdrawal of 500 µl of sample by the injection needle. The injection needle is then placed above on an ABICAP-column by the robotic arm, and the sample is brought on the column. After the injection needle and connective tubes are rinsed with wash buffer by the dilutor, wash buffer is given on the column through the injection needle to remove unbound proteins. This cycle is repeated with elution buffer, whereby the eluted antibodies are captured in test tubes. The eluent is withdrawn out of the test tubes through the injection needle and transported to the injection valve (IV), followed by injection into a carrier flow of elution buffer (0.4 ml min^{-1}). Peak detection is carried out with a fluorescence detector. The procedure was computer controlled. FV = 4-way valve, TV = 3-way valve, P = peristaltic pump.

The maximum peakheight of the sample measurement was normalized with the following equation:

$$NF = \frac{Pmax_{product} - Pmax_{blank}}{Pmax_{BSA}} \quad (1)$$

NF is normalized fluorescence, $Pmax_{product}$ is the maximum peak height of the sample, $Pmax_{blank}$ is the maximum peak height of the blank and $Pmax_{BSA}$ is the maximum peakheight of the BSA solution.

The MAB concentration was calculated with the normalized fluorescence according to a calibration curve. Only one calibration was necessary as a consequence of the endpoint assay and the normalization of the fluorescence signal.

The analysis was started by a signal from the process computer. The analysis cycle, inclusive processing of the peaks, were computer controlled using Datensystem DS450-MT 2 (Kontron, Stuttgart, FRG) as software program. One complete measurement took 6.5 min. The sample frequency was, directly after a change of the temperature, once per hour, and decreased after 8 h gradually to once per day when steady state was reached.

Off-line determinations

Monoclonal antibodies

Off-line measurements of the MAB concentration were carried out with HPLC (2156, Pharmacia LKB, Freiburg, FRG), using a ProAnaMab column (Biolytica, Lund, Sweden). 0.5 ml of sample solution was injected in a 0.11 M sodium acetate binding buffer, pH 5.0. The bound MAB's were eluted with a 0.155 M glycine, pH 1.6 buffer. Peaks were detected at 280 nm with an UV detector. The determined concentrations were corrected

for the antibody concentration in the culture medium by measuring the original medium.

Cell counts

The concentration of cells in suspensions were determined using the erythrosine B exclusion method (Philips, 1973).

The concentration of immobilized cells was determined at the end of each steady state. A sample volume of approximately 0.5 ml micro-carrier was taken out of the reactor. The medium was quickly withdrawn and 2 ml 0.1 M citrate buffer in Phoshate-Buffer-Saline (PBS), pH 7.2 was added. PBS consisted of 137 mM NaCl, 27 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4. The microcarriers were shaken vigorously and incubated at 37 °C. After 1 h of incubation, the cells in suspension were counted with erythrosine B. The microcarriers were washed with water and dried. The volume of the carrier was calculated using a calibration curve (data not shown).

The number of lysed cells was determined indirectly (Wagner *et al.*, 1992) by measuring the activity of lactate dehydrogenase in the medium according to Vassault (1974).

Glutamine

The glutamine concentration was determined by HPLC (Hewlett Packard, Waldbronn, FRG) using derivatisation with o-phtaldialdehyde (Hewlett Packard). Derivatised glutamine and other amino acids were separated on a reverse-phase column (RP-18 Hypersil ODS, Machery and Nagel, Düren, FRG). The amino acids were eluted with a 20 % to 80 % (v/v) gradient of methanol in 0.1 M sodium acetate. Peaks were detected with a fluorescence detector (excitation 230 nm, emission 455 nm).

Precipitation of proteins before analysis was carried out by adding 0.1 ml of trichloroacetic acid (36 % (w/v)) to 0.5 ml of sample. After centrifugation, 15 µl of 4.5 M NaOH was added to 300 µl of supernatant. The sample was further diluted with PBS buffer to the selected concentration.

Ammonium

The reaction of glutamate dehydrogenase as described by Bergmeyer *et al.* (1985) was used to measure the ammonium concentration. Glutamate dehydrogenase (EC 1.4.1.3) type III from bovine liver was purchased from Sigma (Deisenhofen, FRG).

Glucose and lactate

Both the glucose and lactate concentrations were determined with an YSI 2000 automate (Yellow Springs, USA).

Chemicals

All chemicals were of analytical grade. All solutions were made with distilled/de-ionized water.

RESULTS AND DISCUSSION

On-line analysis and sample system

To monitor high-molecular proteins on-line, the interface between the culture and the on-line analysis, the sample system must have no influence on the concentration of the analyte nor endanger the sterility of the culture. In addition, separation of cells and the analyte must be carried out to prevent pollution of the analytical device and/or to prevent interference by intracellular product. For low-molecular compounds, microfiltration membranes are normally applied. The suitability of such membranes for sampling of high-molecular compounds, e.g. monoclonal antibodies, was tested in a preliminary investigation. Several microfiltration membranes were applied within a cross-flow sample module, whereby the monoclonal antibody concentrations before and after filtration were measured. All the tested membranes showed a declining permeability for the antibodies with increasing filtrated volume (data not shown). This change of the filtration characteristics is due to membrane fouling (Schügerl, 1993), which causes a build-up of a protein and/or cell layer on the membrane surface. This layer reduces the effective pore size and consequently lowers the permeability for

high-molecular compounds (Scheper, 1991). Yet, membrane fouling can be avoided by applying high shear forces over the membrane surface. In this way, microfiltration membranes could be used for on-line monitoring of proteins (Stamm *et al.*, 1989, Freitag *et al.*, 1991). This is, however, less desirable for animal-cell cultures as a result of the damaging effect of shear forces on animal cells.

An alternative way to reduce membrane fouling is regular cleaning and/or exchange of the membrane. This is, however, not possible as long as the membrane is in direct contact with the culture. Therefore, an in-line valve was placed between the culture and the membrane (Fig. 2). Contamination of the reactor was prevented by this in-line valve in combination with a chemical barrier. The placement of the filter behind the in-line valve enabled the intermittent cleaning of the filter with the chemical barrier in order to remove protein and/or cell layer without endangering the sterility of the culture. Since the membrane was only used for cell filtration and not as a sterile barrier, a filter with a larger pore size as microfiltration membranes could be applied to diminish size-exclusion effects. Therefore, a depth filter designed to adsorb high quantities of particles was tested. This filter separates the cells and cell debris from the sample across the membrane instead of at the surface as is the case with microfiltration membranes. Regular microscopic control of the filtrate during the culture showed no leakage of cells. A maximum of 75 ml of sample volume (3 samples) could be filtered before the depth filter became blocked. To increase the operational capacity of the filter, several sterilization solutions and additives were tested for their effectiveness in cleaning the filter between sampling. A 0.5 M sulphuric acid solution caused rapid sticking of the filter. A 0.5 M NaOH solution with additional EDTA, to prevent precipitation of carbonate salts, extended the operational capacity. A further increase was achieved by adding an oxidizing agent, potassium peroxodisulphate, to the sterilisation solution. This resulted in an operational capacity of up to 40 sampling cycles (1.0 l sample volume) per filter.

To detect possible retention of the MAB's by the filter, on-line measured data were compared with antibody concentrations measured in off-line samples taken directly out of the reactor. As can be seen in Table 1, the filter showed an unbiased permeability for antibodies after 40 samples, which were taken on-line over a period of 10 d.

Table 1. Testing of the filtration characteristics of the depth filter with time. The MAB concentrations were determined in samples taken directly out of the reactor (before filtration) and on-line (after filtration).

Sample number	Filtrated sample volume l	MAB concentration before filtration mg l^{-1}	MAB concentration after filtration mg l^{-1}
6	0.150	21.5	22.2
14	0.350	20.9	20.0
18	0.450	23.8	23.0
19	0.475	21.7	22.2
30	0.750	20.0	23.9
36	0.900	22.5	20.4
38	0.950	21.2	22.4
40	1.000	22.8	19.9

The ABICAP method combines the high-binding capacity of immobilized antibodies with the very short diffusion pathways in a gel matrix. The short diffusion times result in a fast adjustment of the binding equilibrium between antibody and antigen. All assay designs known for ELISA can be adapted to this method (Hartmann *et al.*, 1993). The ABICAP method was evaluated by comparison of the on-line data with off-line measured MAB concentrations, which were determined by HPLC (Fig. 5). Linear regression between off-line and on-line data resulted in a slope of 0.944 with an intersection at 2.73 mg l^{-1}. The correlation was 0.900.

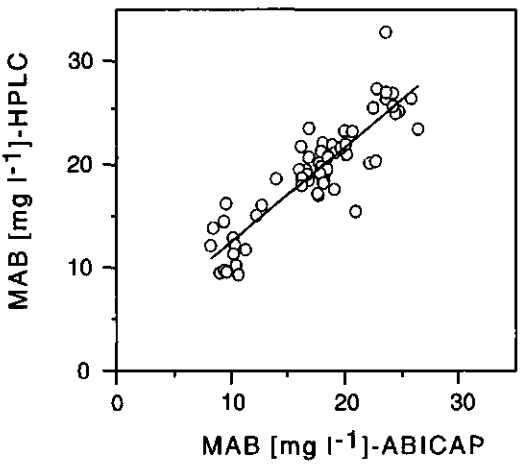


Fig. 5. Comparison of on-line measured MAB concentration (ABICAP) with off-line HPLC measurements. The regression line has a slope of 0.944 with an intersection of 2.73 mg l^{-1}. The correlation is 0.900.

Using a new column of immobilized antibodies for every measurement eliminated any influence that inactivation of the immobilized antibodies may have had on the assay. It also simplified the analysis method considerably, as no labelled antigen or frequent recalibration was necessary. The analytical device functioned without any failures during the culture of 900 h. The fluorescence detector showed a small drift in time, which was corrected by the BSA measurement.

Influence of temperature on the cell metabolism

The relatively high analysis frequency (once per hour) after a temperature change revealed that the MAB concentration can change quickly (Fig. 6). The MAB concentration can also be a sensitive parameter to indicate an alternation of the cell metabolism. Yet, the MAB concentration results from the influence of the environment on the cells and does not elucidate the cause of the change. A good example of this can be seen in Fig. 6, after the temperature was enhanced from 39 °C to 40 °C. The

MAB concentration increased simultaneously with the LDH concentration after this change of temperature. The fast increasing LDH concentration indicates the lysis of many cells (Goergen *et al.*, 1993), which may result in the release of intracellular antibodies and the peak of MAB concentration.

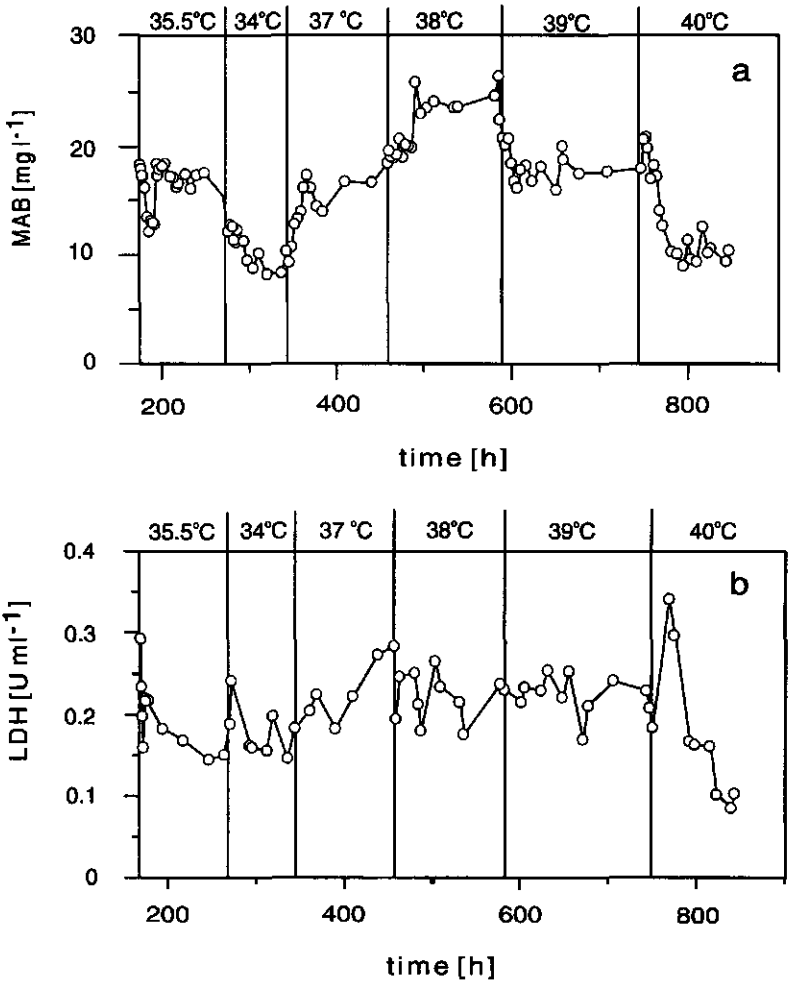
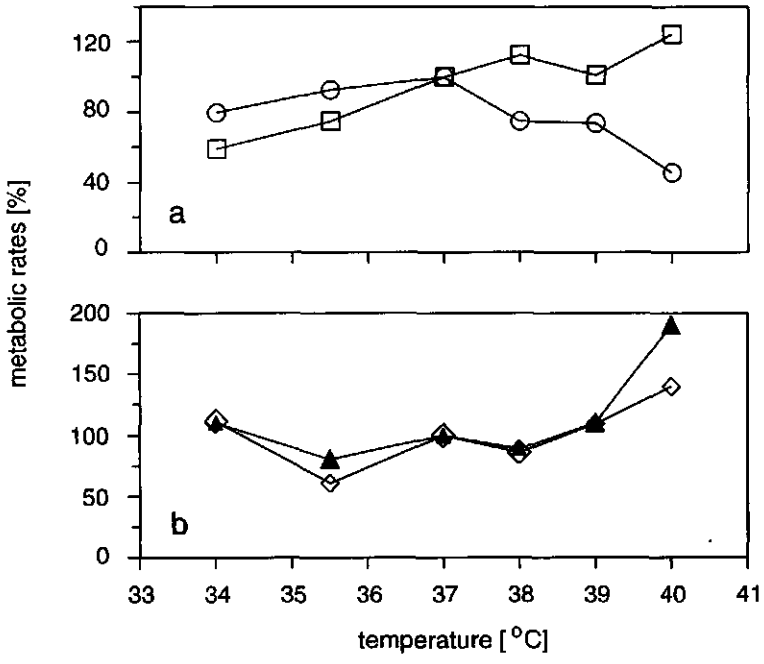


Fig. 6. Course of the concentration of MAB (a) and LDH (b) during culture.

The growth rate was calculated using the mass balance over the reactor for suspension cells released from the microcarriers and lysed cells, and the mass balance of cells over the microcarriers. The calculated growth rates were, however, too high (data not shown). It appeared that with only one incubation with citrate buffer, as used in this study, not all cells were released from the microcarriers. However, the determined immobilized-cell concentration were constant for the temperature range from 34.5 °C to 39 °C. This corresponds with results of Thömmes *et al.* (1993), who also found a constant degree of colonization under different culture conditions. Only at 40 °C, the immobilized-cell concentration decreased significantly by 50 %. Therefore, the trend of the immobilized-cell concentration are considered the same and the growth and other specific metabolic rates are presented as a percentage of the specific rates at 37 °C (Fig. 7).



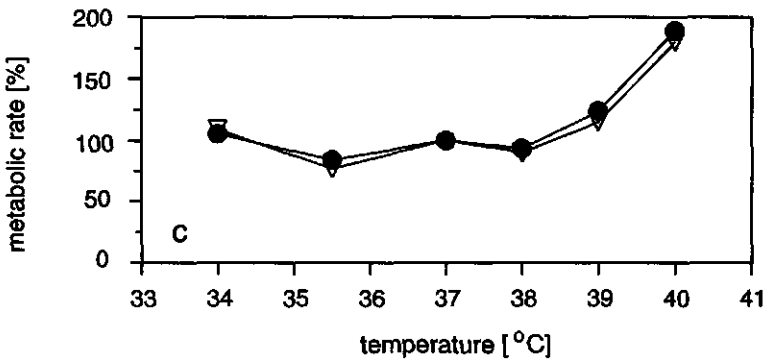


Fig. 7. Steady-state metabolic rates at different temperatures. The metabolic rates are in percentages of metabolic rate at 37 °C (100 %) (a) Growth rate (○) and specific MAB production rate (□). (b) Specific glutamine consumption rate (▲) and specific ammonium production rate (◇). (c) Specific glucose consumption rate (●) and specific lactate production rate (▽).

The specific MAB production and the growth rate simultaneously increased from 34 °C up to 37 °C. A further increase in temperature had the opposite effect, as the growth rate decreased and the specific MAB production further increased to a maximum at 40 °C. Different optimal temperatures for maximum specific production rate and growth rate are also found for suspension cells (Table 2). Barnabé and Butler (1994) suggested that changes in specific nucleotide concentrations and ratios may have an influence on the specific production rate. The maximum MAB concentration of 25 mg l^{-1} was however reached at 38 °C (Fig. 6), because the immobilized cell concentration at this temperature was twice as high as at 40 °C (data not shown).

The specific consumption rate of glucose (q_{glc}) and glutamine (q_{gln}) and the specific production rate of lactate (q_{lac}) and ammonium (q_{amm}) were not influenced by raising the temperature from 34 °C to 38 °C. At higher temperatures up to 40 °C, all four rates increased simultaneously,

which corresponds with data from suspension cultures (Table 2). The yield of moles lactate produced per mole glucose consumed ($Y_{lac/glu}$) stayed constant at 1.31 ± 0.07 in the investigated temperature range, which was also found by Barnabé and Butler (1994), but this contradicts data from Sureshkumar and Mutharasan (1990). The yield of moles ammonium produced per mole glutamine ($Y_{amm/gln}$) consumed showed some, but not consistent variation, and is therefore regarded as constant at 0.35 ± 0.06 for the investigated temperature range. The constant yield of ammonium from glutamine partly agreed with data from Barnabé and Butler (1994), who found a constant yield for the temperature range between 37-39 °C.

Table 2. The temperature (range) at which maximum specific metabolic rates were reached for suspension cultures and immobilized culture.

Cell line	Temp range °C	q_{mab} °C	μ °C	q_{glc} °C	q_{lac} °C	$Y_{lac/glc}$ °C
Suspension cultures						
murine[1]	33-39	39	33-37	39	39	constant
rat-mouse-mouse[2]	34-38	constant	34-37	n.d.	n.d.	n.d.
mouse-mouse[3]	33-39	39	35-39	39	39	39
Immobilized culture						
mouse-mouse	34-40	40	37	40	40	constant

n.d. = not determined, q_{mab} = specific MAB production rate; μ = growth rate; q_{glc} = specific glucose consumption rate; q_{lac} = specific lactate production rate; $Y_{lac/glc}$ = ratio of mol produced lactate to mol consumed glucose.
[1] Barnabé and Butler (1994), [2] agitated cultures, Bloemkolk *et al.* (1992), [3] Sureshkumar and Mutharasan (1990).

CONCLUSIONS

A sample system with a combination of a chemical barrier and cell filtration outside the reactor was successfully used to monitor on-line the antibody production of a hybridoma culture for almost 900 h. The chemical barrier enabled intermittent rinsing of the filter to eliminate the influence of membrane fouling on the permeability for antibodies. Moreover, it enabled a further reduction of size-exclusion effects by the application of a depth filter with larger pore size as a microfiltration membrane. This resulted in a filtration capacity of 1.0 l sample volume (40 samples) or more per membrane. The automated ABICAP method proved to be very reliable and accurate in comparison with HPLC. With a combination of the ABICAP method and the sample system, it will be possible to detect deterioration of the production caused by genetic alteration of the cells. This opens a possibility to intervene in the production process at an early stage to avoid inefficient production.

The maximum specific MAB production rate and the maximum growth rate of the immobilized hybridoma cell line were reached at 40 °C and 37 °C respectively. The maximum specific metabolic rates for glucose, glutamine, lactate and ammonium were reached at 40 °C, whereas $Y_{lac/glu}$ and $Y_{amm/gln}$ stayed constant for the investigated temperature range. The influence of temperature on the specific metabolic rates of immobilized cells and suspension cells was similar.

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Chapter 6

On-line Bioprocess Monitoring and Control using Flow-Injection Analysis

On-line monitoring and control of biotechnological processes will be more frequently applied in industry if suitable analytical systems are available. The results of recent developments in flow-injection analysis means that such analytical systems could be available for use in the near future.

INTRODUCTION

Submerged cultures of cells are characterized by complex interactions between cells and their environment. Detailed information about this environment will enable more insight into the process and its optimization for production. Accurate monitoring of cultures requires high-frequency analysis, which results in high costs when using off-line analysis. In contrast, on-line analysis can drastically reduce manpower requirement and can be used to automatically control the environment. With on-line control, more efficient production and higher consistency in product quality can be achieved (Van der Linden, 1986). Despite these obvious benefits, only a few parameters, such as the dissolved-oxygen and carbon-dioxide concentrations, pH and temperature are controlled on-line during most bioprocesses in industry. Important parameters, such as biomass, product, substrate and inhibitor concentrations are commonly measured off-line at a low frequency and with a long time delay.

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One on-line analysis technique which has been applied for on-line monitoring and control of biotechnological processes in many studies (Table 1) is Flow-Injection Analysis (FIA), a technique introduced by Ruzicka and Hanssen (1975). The frequent utilization of FIA for on-line process analysis is due to the ability to automate a wide variety of analytical assays. The inconsistency between on one side, the frequent utilization of FIA-manifolds in the "academic world", and on the other side the off-line analysis in industry can only be resolved if analytical systems fulfill the following requirements:

1) Analysis of one compound in complex media should be possible (*Selectivity*)
2) Analyte concentrations varying with up to three orders of magnitude must be determined (*Detection range*)
3) Relatively short analysis times (*Analysis frequency*)
4) Changing conditions during a culture should not influence the determination (*Reliability*)
5) The analytical device must be stable and deliver accurate and reproducible results for periods of weeks or months (*Robustness*)
6) Complex maintenance operations should not be necessary, because people carrying out the process in industry will not have the knowledge and/or time to do them (*Maintenance*).

Selectivity can be obtained by applying, for example, enzymatic or immunological assays, which can be easily automated with FIA. To adapt the analytical assay to the conditions of a specific bioprocess, FIA offers automated methods for sample pretreatment to improve reliability, selectivity and to broaden the detection range. Robustness and maintenance will primarily depend on the applied methods in the FIA system, however, software and hardware will also have a substantial impact on these factors.

Table 1. Examples of compounds which have been determined on-line using FIA

Analyte	Method or catalyst	Cultivated organism	Reference
Glucose	Oxydase	Bacteria, yeast	Dullau *et al.*, 1989
L-Lactate	Oxydase	Animal cells	"
Maltose	a-Glucosidase/Glucose oxydase	Fungi	"
Ethanol	Oxydase	Bacteria, Yeast	Garn *et al.*, 1989
Sucrose	Invertase/Mutarotase/ Glucose oxydase	Bacteria	Schügerl *et al.*, 1993.
Antibody	Antibody, Protein G	Animal cells	Schulze *et al.*, 1994.
rt-PA	Antibody	Animal cells	"
D-lactate	Dehydrogenase/Glutamate-pyruvate transaminase	Bacteria	Shu *et al.*, 1995
Acetate	Chemically	Bacteria	Forman *et al.*, 1991
Phosphate	Chemically	Bacteria	"
Penicillin V	ß-Lactamase	Fungi	Carlsen *et al.*, 1993.
Lactose	ß-Galactosidase/Glucose oxidase	Fungi	Schügerl *et al.*, 1991
L-Amino acids	Oxidase	Bacteria	"
Protein	Chemically	Bacteria	Nielsen *et al.*, 1990.
Glucose	Dehydrogenase/Mutarotase	Animal cells	Van der Pol *et al*, 1994
Glutamine	Glutaminase/Glutamate dehydrogenase	Animal cells	"
Lactate	Dehydrogenase	Animal cells	"
Ammonia	Chemically	Animal cells	"

In summary therefore, despite the numerous publications describing on-line monitoring and control with FIA, most of the process-analysis in industry is still done off-line. This manuscript illustrates the possibilities for the application of selective assays and sample pretreatment, which should increase the acceptability of FIA. The application of selective enzymatic assays by which the reaction product is transported to a detector is taken as an example and therefore immuno and chemical assays are not considered here. On-line monitoring using biosensors, whereby the catalyst is in close contact with the transducer, is extensively discussed in the excellent review of Schügerl *et al.* (1996). Although the methods excluded from this manuscript have their own characteristic features, corresponding methods for sample pretreatment will be necessary too. Some aspects of the hardware and software package are also considered in this paper, as they can contribute positively to the robustness and maintenance requirements of FIA-systems.

A necessary and very important component for on-line analysis is the sample system. In this manuscript we assume that a sample system, which delivers a representative, bubble- and cell-free sample from the culture, will be available. For a recent review of sample systems we refer to Mattiasson and Hakanson (1993), who discuss the factors which should be considered in the choice of the sample system.

FLOW-INJECTION ANALYSIS

The instantaneous injection of a sample plug (5-100 µl) into a laminar flow of carrier and/or reagent solution is characteristic of FIA (Fig. 1a). During the transport from the injection valve towards the detector, the sample plug or zone is mixed with the carrier flow by dispersion, causing peak broadening and a lower peak maximum. This results in automatic dilution and conditioning of the sample with the carrier flow. A measure for the dilution is the dispersion coefficient (D), which is the ratio between the concentration of analyte in the sample prior to injection

(C_o in Fig. 1b) and the concentration at the peak maximum of the FIA signal (C_{max} in Fig. 1b; Ruzicka and Hanssen, 1978):

$$D = \frac{C_0}{C_{max}} \tag{1}$$

As long as the carrier flow is constant, the dilution of the sample with the carrier flow and the transport and/or reaction time between the injection valve and the detector will be highly reproducible for every sample injected. Consequently, the analytical reaction must not reach the equilibrium to obtain reproducible measurements, which enables short analysis times and a high analysis frequency.

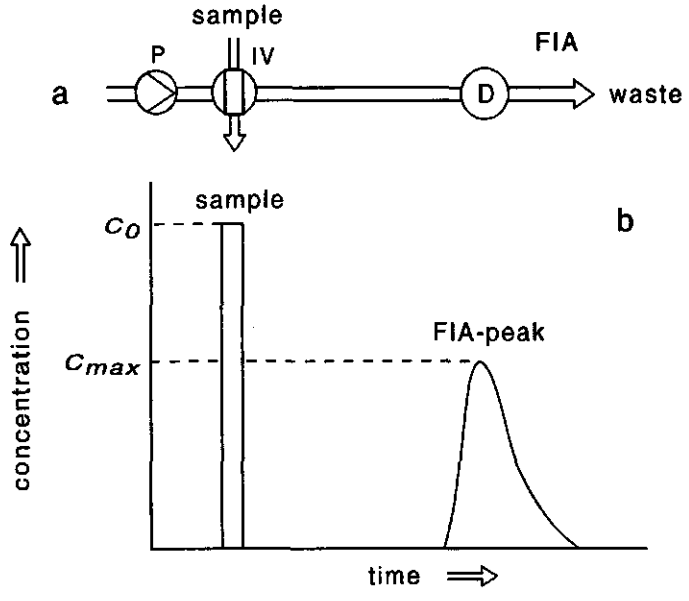

Fig. 1a: Single-line FIA manifold. b: Dispersion of injected sample zone in FIA-manifold. C_0 = concentration in sample before injection, C_{max} = concentration of analyte at the peak maximum P = pump, IV = injection valve and D = detector.

The dispersion coefficient of a single-line flow-injection system, as shown in Fig. 1a, can be manipulated using the parameters of each individual component. The dilution of the sample can be increased by reducing the injected sample volume, increasing the flow rate and by increasing the diameter and length of the connecting channel between injection valve and detector. However, these parameters can only be changed between certain limits. Physical limitations are set by the need to have a minimal injection volume to achieve reproducible injections (Whitman and Christian, 1989) and by the back-pressure in a longer connecting channel. Increasing the length and diameter of the connecting channel will reduce the sample frequency as a result of longer retention time of the samples in the analytical set-up. Moreover, enhancement of the dispersion coefficient in a single-line system will broaden the peaks, lengthening the response time of the detector. Consequently, the time period between two sample injections must be longer. These limitations mean that a dispersion coefficient of maximal 10 is attainable in a single-line configuration under typical conditions for flow-injection analysis (Ruzicka and Hanssen, 1978).

ENZYMATIC ASSAYS INTEGRATED IN FIA-SYSTEMS

The determination of a single analyte in the complex solutions used for bioprocesses is only possible with a highly selective assay. Enzymatic assays are well known for their selectivity and are regularly used for the determination of organic compounds. In FIA-systems, dehydrogenases and oxydases are often applied, whereby the concentration of consumed or produced cofactor is determined (Table 2). For the dehydrogenase reaction, the determination of the analyte is carried out by the detection of NADH. For the oxydase reaction, either the consumed oxygen or the produced peroxide, using a sequential reaction, is detected. However, a linear measurement range with enzymes as catalysts will be only obtained when either the analyte concentration is low compared to the *Km* value of the enzyme for the case of Michaelis-Menten kinetics or when the analyte is completely converted. The latter can only be achieved with high enzyme concentrations.

Table 2. Analytical reactions with dehydrogenases and oxydases

Dehydrogenase reaction

$$\text{Analyte} + \text{NAD} \rightleftharpoons \text{Product} + \text{NADH}$$

Oxydase reaction

$$\text{Analyte} + O_2 \rightleftharpoons \text{Product} + H_2O_2$$

The most direct way to apply enzymes in FIA-systems is as a solution (Fig. 2a). The sample is mixed in the FIA-system with the enzyme solution and the reaction takes place during the transport to the detector. This method was applied by Valero *et al.* (1990) for the on-line monitoring of a yeast culture. However, due to the high consumption of enzyme, analytical costs can be high. A more efficient application of enzymes is achieved by immobilization on either the inner-wall of a nylon tube or on glass carriers. The repetitive use of immobilized enzymes also allows higher catalyst concentrations per measurement, resulting in much faster and/or higher conversions of the analyte. Immobilization on the innerwal of a nylon tube (Fig. 2b) has the advantage that no barrier in the carrier flow exists which could cause fluctuations in the flow rate by variable pressure drops. A disadvantage is the relatively small surface area for immobilization and consequently a low immobilized enzyme concentration in comparison to the glass carriers. This results in relatively low conversion rates.

The immobilization of enzymes on controlled-pore glass carriers is the most frequently applied method (Fig. 2c). As a result of the large surface area, high concentrations of immobilized enzyme are possible. The high enzyme load results in high conversion rates with an efficiency of maximal 100 % (Johansson *et al.*, 1983; Olsson *et al.*, 1986). One disadvantage is that the carrier flow can fluctuate as a result of a variable pressure drop over the enzyme reactor. However, the pressure drop is low and stays constant when carriers with diameter in the range of 125-200 µm are used (Dullau *et al.*, 1989). Another disadvantage of applying immobilized enzymes is that inactivation over time can occur, which causes a deterioration of the conversion rate and a concurrently lower signal.

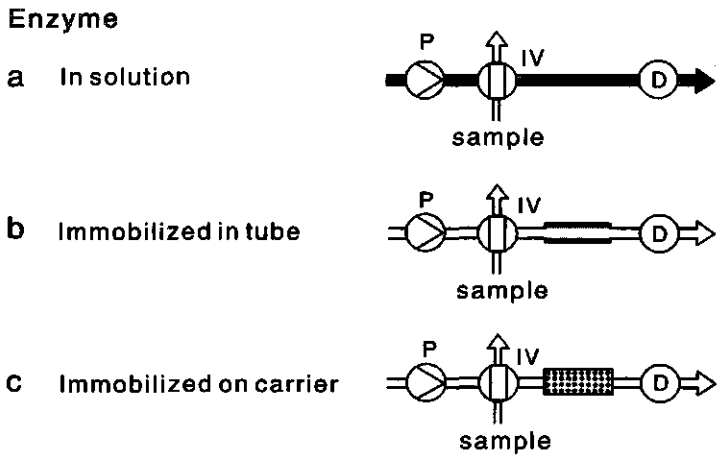

Fig. 2. Application of enzymes in FIA-systems. a: Enzyme solution is pumped through the FIA-manifold. b: The enzyme is immobilized on the inner-wall of a nylon tube. c: The enzyme is immobilized on controlled-pore glass carriers. P = pump, IV = injection valve and D = detector.

A general disadvantage of enzymatic assays is that they are susceptible to interference from physicochemical features of the sample such as pH and temperature and to interference from components in the sample such as ions and inhibitors. An illustrative example of this interference is shown in Fig. 3, where chloride ions, which are commonly present in media, affected the determination of glutamate with glutamate dehydrogenase. This was also found for an alcohol oxidase (Jürgens *et al.*, 1994).

SAMPLE PRETREATMENT USING FLOW-INJECTION ANALYSIS

The adaptation of an analytical assay to a specific bioprocess is mainly based on dilution of the sample with a buffer solution to increase the detection range, to obtain a linear measurement range (see enzymes) and to suppress interference of other components in the sample. For many samples of bioprocesses, higher dilution rates, such as can be reached with single-

line FIA systems, are required for on-line analysis. Therefore, more complicated and faster dilution methods were introduced into FIA-systems. Some examples of methods which are often applied are discussed below and are illustrated in Fig. 4. For a more complete overview of FIA-methods in general, we refer to Ruzicka and Hanssen (1988) and Clark *et al.* (1990).

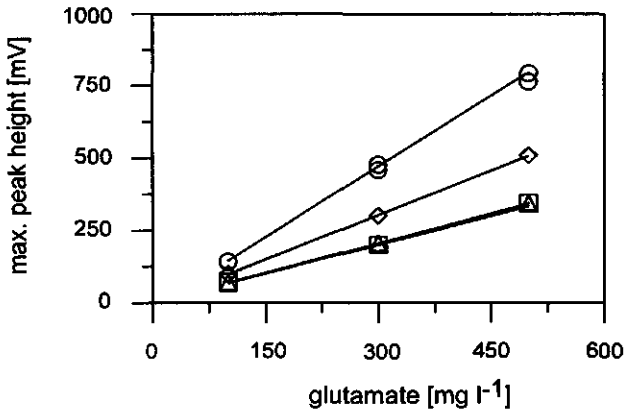

Fig. 3. Influence of Cl^- ions on the maximum peak height of a glutamate assay, using glutamate dehydrogenase immobilized on controlled-pore glass carriers. The glutamate solution (0.4 g l^{-1}) was dialysed in the FIA manifold. The acceptor flow was mixed after the dialysis cell with a carrier flow supplemented with the salts. Concentrations of the salts are the concentrations after mixing of the two carrier flows. (○) no salt addition, (◇) 0.1 M KCl, (□) 0.2 M KCl, (△) 0.1 M $CaCl_2$.

Stream-splitter method (Fig. 4a)

This method is based on the reduction of the sample volume to below the minimum sample volume of the injector valve, without influencing the concentrations in the sample zone. The carrier flow, which also contains the sample zone, is continuously split, whereby one half is directed to the detector, the other leaves the analytical system. The final dispersion coefficient of the analytical system is affected by the volume of the

remaining sample zone and is therefore influenced by the ratio of the two flows. Applying this method, Valero *et al.* (1990) enhanced the determination range for the glucose concentration threefold and were able to measure between 0.8 - 90.0 g l^{-1} during a yeast culture. A disadvantage of this method is the high consumption of carrier solution.

Split-zone method (Fig. 4b)

This method is also based on the reduction of the sample zone after injection, however, a part of the sample zone is cut out instead of divided. After injection, the sample zone is propelled to the confluence tee by pump P1. When a certain fraction of the sample zone has passed the tee, pump P1 is stopped and valve V is closed. By starting pump P2, the fraction of the sample zone between the tee and injection valve is propelled into the FIA system. The active time of pump P1 determines the residual amount of sample and consequently the corresponding dilution. Dispersion coefficients with a maximum value of 300 with a Relative Standard Deviation (RSD) of 3 % or less could be achieved using a similar system (Clark *et al.*, 1989). More complex systems can reach higher dispersion coefficients. In comparison to the stream-splitter method, this method consumes much less carrier solution, however, it requires very precise timing.

Zone-sampling method (Fig. 4c)

Zone-sampling is based on the injection of a zone of an exponentially decreasing gradient of the sample, which is produced by a stirred-mixing chamber (smc). It is possible to select the required dilution by varying the time of the second injection, which can also be changed during a culture. Providing that the mixing in the chamber is homogeneous, the dispersion coefficients attainable vary from a minimum of 20 up to 1500 with a RSD as low as 0.5 % (Gisin and Thommen, 1986). Other advantages are the relatively short time periods needed to achieve a high dilution and the insensitivity to the viscosity of the sample. The zone-sampling method was applied by Garn *et al.* (1989) to the on-line monitoring of cultures of several micro-organisms. A disadvantage of this method is the time required to wash the gradient out of the chamber, before a new sample can be injected.

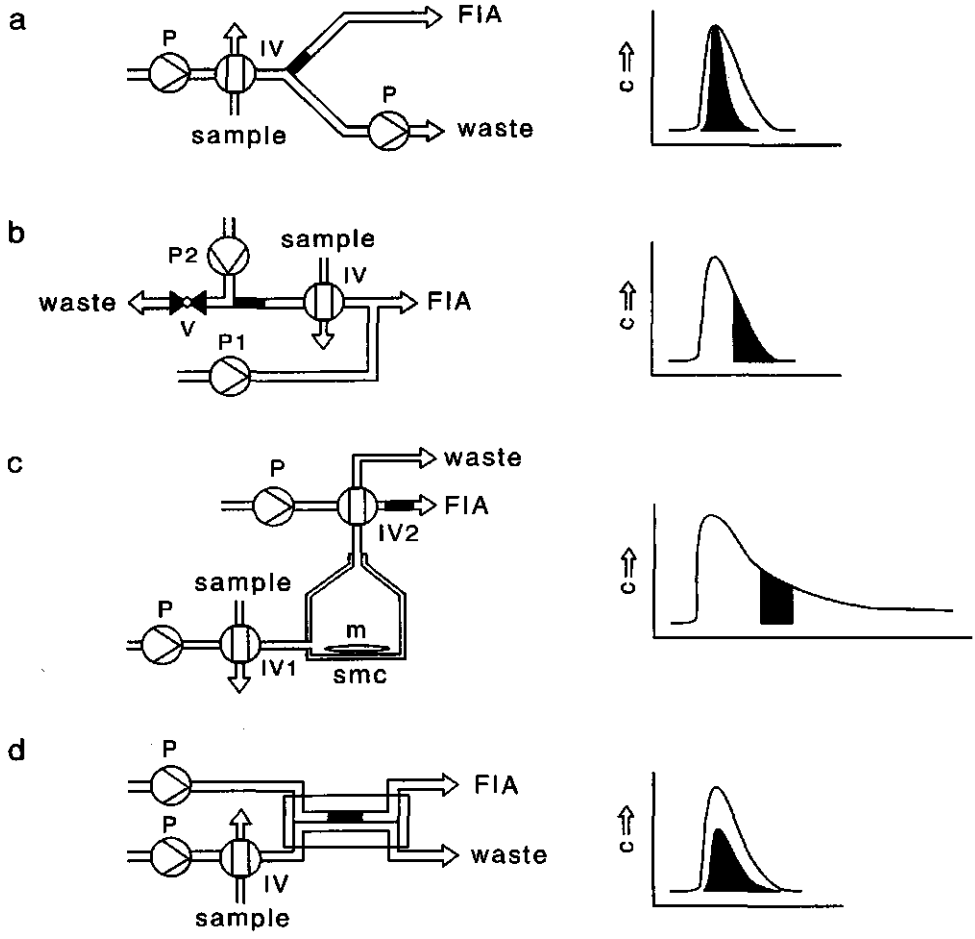

Fig. 4. Methods to obtain relatively high dilution rates of the sample in FIA systems. The hatched part of the peaks is used for the analysis. (a) Stream-splitter. The carrier flow containing the sample zone is continuously split, whereby one part is aspirated into FIA-system, the other part leaves the analytical system. (b) Split-zone. A part of the sample zone is cut out, before aspiration into the FIA-manifold. (c) Zone sampling. A fraction of the outlet of a stirred-mixing chamber is injected into the FIA-system. (d) Dialysis. The analyte is separated from the sample and diluted by dialysis over a membrane. P = pump, IV = injection valve, V = valve, smc = stirred-mixing chamber and m = magnetic stirrer.

Dialysis (Fig. 4d)

Dialysis combines dilution with the selective removal of interfering substances. Both the donor, containing the sample, and acceptor solutions are propelled through small channels separated by a membrane. The dilution achievable is influenced by the configuration of the channels, the flow rates of donor and acceptor solution, the membrane and its surface area and physicochemical parameters (see below). Dilution ranges vary from approximately 10 up to 400 times (Van Staden and Van Rensburg, 1990). Determinations with flow-injection systems using dialysis had standard deviations below 1 % (Olsson *et al.*, 1985; Van Staden, 1992; Künnecke and Schmid, 1990).

The membrane acts additionally as a selective barrier, that separates the analyte from other compounds in the sample. Membranes with a low cut-off separate low-molecular from high-molecular substances or particles, which could interfere with detection or block connecting channels. Hydrophobic membranes, which are not hydrophilized, can only be traversed by volatile compounds. In this way, the selectivity of assays for compounds as for example ammonia and alcohols can be enhanced. A disadvantage of dialysis is that the separation efficiency is influenced by salt concentrations (Fig. 5), protein concentration and osmotic pressure differences between donor and acceptor solution (Van Staden and Van Rensburg, 1990), pH, viscosity and temperature. Therefore, sample pretreatment must be carried out prior to the dialysis, to suppress the influence of these factors.

ROBUSTNESS AND MAINTENANCE OF FIA SYSTEMS

The most important parameter for the reproducibility of analysis with FIA is a constant flow rate. Only constant flow rate will give highly reproducible dispersion coefficients and reaction times for all processes carried out. Therefore, the robustness of a FIA-system is highly dependable on the stability of the pump which generates the carrier flow. Careful selection between peristaltic, piston or HPLC pumps in relation to the applied methods in the FIA manifold should be made. Peristaltic pumps are

the cheapest, but produce a pulsed flow and are less constant in time than the other types. Piston pumps are more precise than peristaltic pumps and pulse free, but are more expensive and susceptible to pressure. The most reliable, but also the most expensive pumps, are HPLC pumps. One way to reduce the influence of the carrier flow is by achieving a 100 % enzymatic conversion of the analyte (Johansson *et al.*, 1983). This can be accomplished either by stopping the carrier flow during the analytical reaction (stopped-flow) or by applying a surplus of catalyst capacity (immobilization). Stopped-flow results in longer reaction times, but has the advantage that higher conversion rates are achieved with negligible peak broadening.

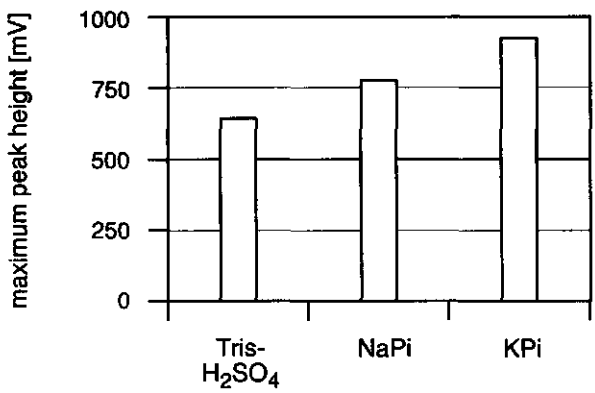

Fig. 5. Influence of positive ions on the transfer rate of dialysis with a membrane of regenerated cellulose. The salts were added to a glutamate solution and pH was adjusted to 7.3. The glutamate solution was continuously dialysed, and the acceptor solution was injected into a second carrier flow. The second carrier flow transported the sample to an immobilized glutamate dehydrogenase, which converted the glutamate to α-ketoglutarate. The simultaneous build NADH was detected fluorimetriccaly. The maximum height of the NADH-peak is a mass for the separation efficiency. KPi = potassium phosphate, NaPi = sodium phosphate and Tris-H_2SO_4 = tris-(hydroxymethyl)-aminomethane, whereby the pH was adjusted with sulphuric acid.

Another important factor, which can contribute to the robustness and acceptance of FIA-manifolds in general is "intelligent" software. The software should not only be able to control the hardware, but also be able to perform automatic calibration, peak processing and failure diagnosis of peaks and hardware. An example is the software package called FIACCO (Flow-Injection Analysis Control and Configuration), which was developed in a cooperation between the University of Halle-Wittenberg (FRG) and the Forschungszentrum Jülich GmbH (FRG) (Spohn *et al.*, 1994). This program can control up to six independent analysis channels. To enable the adaptation of the assays to the bioprocess, a high number of different valves, pumps and detectors can be controlled in separated time courses with a time resolution of 0.5 s. The program automatically executes peak processing, calculation of the detection limit and calibration, enabling the calibration graphs to be calculated according to different regression methods. Several digital filters are available to improve the signal/noise ratio of the detector output. The functioning of valves and pumps is electronically diagnosed by the software.

A very important feature to improve the robustness of the analytical system is recalibration. This is required to check if the calibration curve used to calculate the concentrations is still valid. Since a complete calibration will result in a long period without process analysis, one- or two-point recalibration is preferred. As long as the result of the recalibration lies between preadjusted borders, no further action is taken. When the result of the recalibration is unsatisfactory, a completely new calibration is executed. During the on-line monitoring of an animal-cell culture for almost 1000 h, daily recalibration was used to correct for enzyme inactivation. This resulted in extended operational times for the applied enzyme reactors up to 4500 analysis (Van der Pol *et al.*, 1994). This experiment and others (Van der Pol *et al.*, 1995; Blankenstein *et al.*, 1994) have shown that FIACCO is suited for on-line monitoring and control of bioprocesses. However, further improvements can be made to enhance the robustness and facilitate the acceptance of FIA systems in industry by applying supervisory programs. Filippini *et al.* (1992) used a supervising computer to adapt the parameters of the analytical system, e.g. dilution and standard concentration for

recalibration, automatically to the actual state of the process. A further way to improve robustness is the application of an interactive expert system, which can diagnose malfunctioning of the FIA-system based on differences between the complete measured signal and an ideal signal (Brandt and Hitzmann, 1994). A significant advantage of such a knowledge-based system is that it can indicate what is likely to cause the failure, which enables inexperienced operators to quickly neutralise the cause of the malfunction. Other advisory expert systems can assist in the quantitative validation of FIA-assays (Wolters *et al.*, 1990).

CONCLUSIONS

Flow-injection analysis is now a mature method for the on-line monitoring and control of biotechnological processes. The necessary *selectivity* to carry out analysis in media of bioprocesses is obtained by the application of selective catalysts in FIA systems. *Detection range, analysis frequency* and *reliability* can be optimized towards a particular bioprocess by sample pretreatment. The *robustness* and *maintenance* can be improved by application of stable hardware and "intelligent" software. Therefore, FIA has the potential to make on-line monitoring and control feasible for day to day use in industry.

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Summary

On-line analysis and control of biotechnological processes is still the stepchild in industry. In general, only parameters as dissolved-oxygen concentration, pH and temperature are controlled on-line. Important parameters as substrate and inhibitor concentrations are only measured off-line. Controlling these parameters could not only result in more efficient production and higher product quality, but could also save manpower. The attitude in industry could change if analytical systems were available which are selective, reliable and robust, have low maintenance requirements and a large detection range (chapter 6 of this thesis). However, such systems are commercially seldom available. Therefore, an analytical system based on the flow-injection analysis (FIA) method was developed, which could fulfil the requirements mentioned above. The possibilities of this new analytical system are described in chapter 2 of this thesis. A software program (FIACCO) was developed which could control a high amount of hardware components and was able to carry out peak evaluation, automatic (re)calibration and outlier tests. This program in combination with precisely working pumps enabled the automatic and reproducible application of almost every known FIA method for sample pretreatment as dilution, pH adjustment and the elimination of interfering substances. Up to five analytes could be determined with the analytical system using enzymatic and/or chemical assays and only one detector.

The developed analytical system was applied for the on-line monitoring of the glucose, glutamine, lactate and ammonium concentration during the culture of animal cells, which is described in chapter 3 of this thesis. Although some small disturbances occurred during a long term culture of 1000 h, the system proved to be reliable and robust and had very low maintenance requirements. The high analysis frequency could be used to estimate the growth rate during start-up periods of the culture. Moreover, it showed that in contradiction with general opinion, animal cells can react quickly to changes in the environment.

The on-line control of animal-cell cultures by using the analytical system is described in chapter 4 of this thesis. The closed-loop control was realized by the application of an Extended Kalman filter and a minimum variance controller. Cultures could be started up at either a constant glutamine or glucose concentration, whereby the perfusion rate was adapted automatically to the increasing consumption rate of the controlled substrate. Glutamine was the better control substrate for the increasing perfusion rate with respect to avoiding substrate limitations. During a culture period of more than 800 h, the glucose and glutamine concentrations were controlled independently at different perfusion rates. The concentrations deviated in general maximally 10 % of the setpoint.

Since the product concentration and/or productivity are also important process parameters, an analytical setup was developed for the on-line monitoring of the product concentration. A commercially available immunoassay (ABICAP) was coupled to an animal-cell culture by a newly developed sample system, which is described in chapter 5 of this thesis. The concentration of product, a monoclonal antibody, was monitored successfully for nearly 900 h. During the culture, the specific monoclonal antibody production rate increased with increasing temperature of the culture.

Samenvatting

Het automatisch analyseren en regelen van biotechologische processen is nog steeds een stiefkind in de industrie. In het algemeen worden alleen de zuurstofconcentratie in het medium, de zuurgraad (pH) en de temperatuur automatisch gemeten en geregeld. Belangrijke grootheden als de concentraties van substraten en remmers worden alleen met de hand bepaald. Het automatisch regelen van deze grootheden zou niet alleen een efficiëntere procesvoering en een betere produktkwaliteit mogelijk maken, maar kan ook personeelkosten besparen. De industriële attitude zou kunnen veranderen wanneer meetapperatuur beschikbaar komt die selectief meet, betrouwbaar en robuust is, weinig onderhoud nodig heeft en een groot meetbereik heeft (hoofdstuk 6 van dit proefschrift). Omdat meetapperatuur die aan deze voorwaarden voldoet nog zelden commercieel verkrijgbaar is, is een analyse apparaat op basis van de Flow-Injection Analysis (FIA) methode ontwikkeld dat voldoet aan deze voorwaarden. De mogelijkheden van dit apparaat zijn beschreven in hoofdstuk 2 van dit proefschrift. Er werd een computerprogramma (FIACCO) ontwikkeld, dat in staat is meerdere soorten kleppen, pompen en detectoren aan te sturen, data van de detector te evalueren, automatisch te kalibreren en foutieve metingen uit te sorteren. Met een kombinatie van dit programma en precieze pompen kunnen bijna alle bekende FIA-methodes voor monstervoorbehandeling als verdunnen, verandering van de zuurgraad en verwijderen van storende elementen, automatisch en reproduceerbaar uitgevoerd worden. Met gebruik van chemische en enzymatische analyse methoden en één detector zijn met het meetapparaat tot vijf stoffen tegelijk gemeten.

Het ontwikkelde meetapparaat is voor het automatisch meten van de glucose-, glutamine-, lactaat- en ammoniumconcentratie tijdens de kweek van dierlijke cellen ingezet. Dit wordt beschreven in hoofdstuk 3 van dit proefschrift. Hoewel tijdens metingen gedurende een periode van bijna 1000 uur kleine onregelmatigheden optraden, werd bewezen dat het

meetapparaat betrouwbaar en robuust is, en weinig onderhoud nodig heeft. De hoge frequentie van de metingen maakte een schatting van de groeisnelheid tijdens het opstarten van de kweek mogelijk. Bovendien werd aangetoond dat in tegenstelling tot wat in het algemeen gedacht wordt, dierlijke cellen snel kunnen reageren op veranderende omstandigheden.

De automatische controle van dierlijke celcultures met gebruik van het meetapparaat is beschreven in hoofdstuk 4 van dit proefschrift. Het regelsysteem bestond uit een Extended Kalman filter en een minimum variance regelaar. De kulturen waren opgestart met of een constante glucoseconcentratie of een constante glutamineconcentratie, waarbij de toevoer van het medium automatisch werd aangepast aan het stijgende verbruik van het geregelde substraat. Om een lagere groeisnelheid als gevolg van een te lage toevoer van medium te vermijden, kan beter op glutamine dan op glucose worden geregeld. Tijdens een andere kweek met verschillende toevoersnelheden van het medium, werden de glucose- en glutamineconcentratie onafhankelijk van elkaar gedurende meer dan 800 uren geregeld. De concentraties van glucose en glutamine weken in de regel niet meer dan 10 % van de gewenste concentratie af.

Omdat de productconcentratie en/of productiviteit belangrijke procesparameters zijn, werd een meetsysteem ontwikkeld dat de produktconcentratie in een dierlijke celkweek automatisch kan meten. Een commercieel verkrijgbare analyse methode (ABICAP) werd hiervoor gebruikt. Met deze methode wordt gebruik gemaakt van antilichamen die op een kleine kolom geïmmobiliseerd zijn. De monsters werden met een nieuw ontwikkeld monsternamesysteem, wat beschreven is in hoofdstuk 5 van dit proefschrift, automatisch uit de reactor genomen. De concentratie van het product, een monoclonaal antilichaam, werd gedurende bijna 900 uur automatisch gemeten. Tijdens de kweek bleek dat de productie van antilichamen per cel steeg met stijgende termperatuur.

Zusammenfassung

Die online Analyse und Kontrolle von biotechnologischen Prozessen ist noch immer ein Stiefkind der Industrie. Im allgemeinen werden nur Parameter wie Konzentration des gelösten Sauerstoffs, pH und Temperatur online kontrolliert. Einflußreiche Parameter, wie Substrat- und Inhibitorkonzentration werden nur offline bestimmt. Online Kontrolle dieser Parameter würde nicht nur eine effizientere Prozeßführung und höhere Produktqualität ermöglichen, sondern auch niedrigere Personalkosten. Die Haltung der Industrie gegenüber online Kontrolle könnte sich ändern, wenn Analysengeräte zur Verfügung stünden, welche selektiv messen können, zuverlässig und robust sind, wenig Wartung brauchen und einen großen Meßbereich haben (Kapitel 6). Bisher sind solche Geräte kaum kommerziell verfügbar. Daher wurde ein automatisches Analysengerät auf Basis der Fließinjektionsanalyse (FIA) Methode entwickelt, das den zuvor erwähnten Anforderungen genügt (Kapitel 2). Ein Computerprogramm wurde entwickelt, welches in der Lage ist, eine Vielzahl von Pumpen, Ventilen und Detektoren zu steuern, automatische Kalibrierungen und Ausreißertests durchzuführen. Dieses Programm in Kombination mit präzisen Pumpen ermöglichte die automatische und reproduzierbare Anwendung fast aller bekannten FIA-Methoden zur Probenaufbereitung, wie Verdünnung, Änderung des pH-Wertes und die Entfernung störender Substanzen. Unter Verwendung von chemischen und enzymatischen Analysemethoden konnten mit nur einem Detektor bis zu fünf Substanzen online gemessen werden.

Das neuentwickelte Analysensystem wurde zur online Messung von der Glukose-, Glutamin-, Laktat- und Ammoniumkonzentration einer tierischen Zellkultur angewendet (Kapitel 3). Obwohl kleine Unregelmäßigkeiten während einer Kultivierung von fast 1000 Stunden auftraten, wurde gezeigt, daß das Analysensystem zuverlässig und robust ist und wenig Wartung braucht. Die relativ hohe Analysenfrequenz konnte zur

Schätzung der Wachstumsrate während der Anlaufphase der Kultivierung angewendet werden. Auch zeigte die hohe Frequenz, daß, im Gegensatz zur allgemeinen Meinung, tierische Zellen schnell auf Änderungen der Kulturbedingungen reagieren.

Die online Kontrolle mehrerer tierischer Zellkulturen mit Hilfe des Analysengeräts wird in Kapitel 4 dieser Doktorarbeit beschrieben. Der geschlossene Regelkreis bestand aus einem Kalman-Filter und einem Minimalvarianz-Regler. Die Kulturen wurden entweder mit einer konstanten Glukosekonzentration oder mit einer konstanten Glutaminkonzentration angefahren. Die Mediumzufuhr wurde durch den Reglerkreis dem steigenden Verbrauch des kontrollierten Substrats angepaßt. Es zeigte sich, daß Glutamin in Bezug auf Vermeidung von Substratlimitierung ein besserer Kontrollparameter war als Glukose. Während einer dritten Kultivierung mit unterschiedlichen Durchflußraten, wurden die Glukose- und Glutaminkonzentration unabhängig voneinander für über 800 Stunden geregelt. Im allgemeinen wichen die Konzentrationen nicht mehr als 10 % von der Stellgröße ab.

Weil die Produktkonzentration und die Produktivität wichtige Prozeßparameter sind, ist eine Meßanordnung für die online Messung der Produktkonzentration aufgebaut worden. Eine kommerzielle, immunologische Meßmethode (ABICAP), wurde mit einem neuentwickelten Probenahmesystem an eine tierische Zellkultur angekoppelt (Kapitel 5). Die Konzentration des Produktes, ein monoklonaler Antikörper, ist über eine Zeitperiode von fast 900 Stunden erfolgreich aufgenommen worden. Die Menge an produziertem Antikörper pro Zelle erhöhte sich mit steigender Temperatur der Kultur.

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Curriculum Vitae

Jens van der Pol werd op 24 april 1965 geboren in Velsen. In 1983 werd het diploma OVWO behaald aan het Bonhoeffer College in Castricum. Aansluitend begon hij met de studie Biologie aan de toenmalige Landbouwhogeschool Wageningen. Tijdens deze studie specialiseerde hij zich in de biotechnologie met de afstudeervakken industriële microbiologie, biochemie en proceskunde. Zijn praktijktijden bracht hij door bij de sectie proceskunde, vakgroep levensmiddelentechnologie, Landbouwuniversiteit Wageningen bij Prof. Tramper en bij Ciba-Geigy, Basel, bij Dr. Ghisalba. Het doctoraal examen haalde hij in 1989.

De biologiestudie werd direkt gevolgd door een promotie onderzoek aan het Institut für Biotechnologie, Forschungszentrum Jülich, Duitsland, bij Prof. Wandrey en dat werd begeleid door Prof. Tramper. De resultaten van dit onderzoek staan in dit proefschrift beschreven. In februari 1994 was het praktische werk van dit proefschrift afgesloten, en begon hij een post-doctoraal onderzoek aan het Nestlé Centre du Recherche, Lausanne, Zwitserland bij Dr. nat. ref. Niederberger voor een periode van één jaar. In de periode van februari 1995 tot augustus 1995 werd dit proefschrift geschreven. Vanaf augustus 1995 is hij werkzaam als project manager bij Nestlé R&D Center Orbe, Orbe, Zwitserland.

Lebenslauf

Jens van der Pol wurde am 24 April 1965 in Velsen, Niederlande geboren. Im Jahre 1983 erhielt er das Abschlußzeugnis des Neusprachlichen Gymnasiums Bonhoeffer College in Castricum, Niederlande. Anschließend startete er mit dem Biologiestudium an der jetzigen Landwirtschaftsuniversität Wageningen. Während dieses Studiums spezialisierte er sich mit Studienarbeiten im Bereich der industriellen Mikrobiologie, Biochemie und Verfahrenstechnik auf dem Gebiet der Biotechnologie. Er absolvierte seine praktische Zeit bei Herrn Prof. Dr. Tramper, Abteilung Bioprozesstechnolgie, Fachgruppe Lebensmitteltechnologie, Landwirtschaftsuniversität Wageningen, Niederlande, und bei Herrn Dr. Gishalba, Ciba-Geigy, Basel, Schweiz. Das Studium wurde im Jahre 1989 erfolgreich abgeschlossen.

Dem Biologiestudium folgte diese Doktorarbeit bei Herrn Prof. Dr. Wandrey, Institut für Biotechnologie, Forschungszentrum Jülich. Begleitet wurde diese Arbeit von Herrn Prof. Dr. Tramper. Im Februar 1994 wurde die praktische Arbeit der Promotion abgeschlossen, und er absolvierte für ein Jahr eine Postdoktorandenzeit an dem Nestlé, Centre du Recherche, Lausanne, Schweiz. Während der Zeit von Februar 1995 bis August 1995 wurde diese Dissertation verfasst. Ab August 1995 ist Jens van der Pol als Projektmanager bei Nestlé R&D Center Orbe, Schweiz tätig.