# Serum- and protein-free media formulations for the Chinese hamster ovary cell line DUKXB11

Revised Version

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**Running title:** Serum- and protein-free media for CHO DUKXB11 cells

**Abstract**

The production of therapeutic proteins in mammalian cell lines is of outstanding importance. The maintenance of most mammalian cell lines in culture requires the addition of serum to the culture medium. The elimination of serum from mammalian cell culture is desirable since serum is expensive and a source of contaminants, e.g. viruses, mycoplasma or prions. Here we describe the composition of serum- and protein-free media for the Chinese hamster ovary (CHO) cell line DUKXB11. The serum-free formulation supports excellent growth of CHO DUKXB11 cells at low (23 cells/$cm^2$) and high ($2 \cdot 10^4$ cells/$cm^2$) seeding densities characterized by a generation time of 10 - 12 h, and, after addition of 0.2% pluronic F-68, the growth of a recombinant suspension cell line derived from DUKXB11. In addition, this formulation also allowed us to adapt recombinant cell lines expressing various amounts of human antithrombin ATIII (ATIII) to serum-free conditions. Secretion of ATIII was readily observed in the serum-free medium. Minor changes to the serum-free formulation resulted in a protein free formulation that supported growth of CHO DUKXB11 cells, growth of recombinant CHO cells expressing ATIII, and production of ATIII.

**Key words**

Chinese hamster ovary cells, serum-free medium, protein-free medium, recombinant protein production, antithrombin III

**Abbreviations**

ATIII - antithrombin III, CHO - Chinese hamster ovary, DHFR - dihydrofolate reductase, ELISA - enzyme-linked immunosorbent assay, FCS - fetal calf serum, L-LDH - L-lactate dehydrogenase, MTX - methotrexate

**Introduction**

Genetically engineered CHO cells are an important source for a large variety of proteins of great therapeutic value (Kane, 1991; Kaufman, 1993; Kellems, 1991). A common method to construct recombinant CHO cell lines is to transfect dihydrofolate reductase (DHFR) negative ($dhfr^-$) CHO cell lines with the cDNA genes for DHFR and the protein of interest. Initial transformants are selected for growth in the absence of glycine, purines, and thymidine. The transfected genes are then amplified by stepwise

increasing the concentration of methotrexate (MTX), a competitive inhibitor of DHFR, in the culture medium (Kaufman, 1990). During this process the transfected genes are amplified several 1000-fold resulting in an increased production rate for the recombinant protein (Crouse et al., 1983). These genetic manipulations are usually done in the presence of animal serum. The use of serum is required to grow many mammalian cell lines *in vitro* (Maurer, 1986; Sato, 1975). However, the elimination of serum from the culture process is desirable, since it is expensive, a source of contaminants (Kimberlin, 1991, Minor, 1994), and weaning of recombinant cells from serum is time consuming and unreliable.

Two dhfr$^-$ CHO cell lines are in use today. In DG44 cells both DHFR loci are deleted (Urlaub et al., 1983). A serum-free formulation for this cell line was described (Kim et al., 1999). DUKXB11, also referred to as DXB11, DUKX, DUKXB1, or DUK-XB11 (Gandor et al., 1995), was derived from CHO K1 cells (Urlaub and Chasin, 1980). Clonal growth of CHO K1 cells was reported in the serum-free media F12 (Ham, 1965), MCDB 301, and MCDB 302 (Hamilton and Ham, 1977). However, these media did not support the growth of high-density cultures, and are therefore of limited use for high-level production of recombinant proteins. Serum-independent suspension cell lines, e.g. CHO SSF3, were selected from DUKXB11 cells by replacing part of the medium every week (Gandor et al., 1995). A protein-free medium for CHO SSF3 cells was described (Zang et al., 1995). In addition, a cell line preadapted to serum-free conditions, derived from DUKXB11 cells was described (Sinacore et al., 1996). However, the serum-free medium formulation used in this study was not published. In addition, many formulations for recombinant CHO cells expressing a variety of proteins are described (Asakura et al., 1992; Blüml et al., 1994; Hata et al., 1992; Ii et al., 1995; Kim et al., 1998; Kitano, 1994; Lee et al., 1999; Matthews et al., 1994; Mizuguchi et al., 1993; Scharfenberg and Wagner, 1995; Schorn et al., 1994; Wyatt, 1994). However, a versatile serum-free medium formulation for the parental cell line DUKXB11 has not been reported to date.

Here we present a serum-free medium for CHO DUKXB11 cells that equally well supports growth at low (23 cells/cm$^2$) and high ($2{\cdot}10^4$ cells/cm$^2$) seeding densities. We evaluated the growth properties, production of a model recombinant protein by recombinant CHO cells derived from DUKXB11, and the ease with which minor modifications of this formulation support growth in suspension or protein-free

conditions. We discuss the influence of cholesterol containing growth supplements on growth under serum-free conditions.

## Materials and Methods

### *Materials*

The dhfr$^-$ CHO cell line DUKXB11 was described by Urlaub and Chasin (1980). The ATIII secreting cell lines CHO-A11-A2, CHO-A11-A27, CHO-A11-A279, and CHO-A11-A279-C7 were derived from CHO DUKXB11 cells and secrete increasing amounts of ATIII (Schröder and Friedl, 1997a; Zettlmeissl et al., 1987). The cell line CHO-SS3-A2 (Tebbe et al., 1995) was a kind gift of Prof. J. Lehmann (University of Bielefeld, Bielefeld, Germany). The suppliers of most materials were listed previously (Schröder and Friedl, 1997a). Pluronic F-68 was purchased from Sigma (Deisenhofen, Germany) and casein peptone soybean flour peptone broth from Unipath (Wesel, Germany). A 6 x stock solution of the casein peptone soybean flour peptone broth was filter sterilized and than added to the final medium. Human fibronectin was isolated from human blood plasma (German Red Cross, Frankfurt/Main, Germany) as described before (Schröder and Friedl, 1997a). Animal-source derived components used in the serum-free formulation were fetuin from fetal calf serum (no. 104874, ICN Biomedicals, Eschwege, Germany), insulin from bovine pancreas (no. I-5500, Sigma), human *holo*-transferrin (no. OTRE 04105, Behringwerke AG, Marburg, Germany), and casein peptone soybean flour peptone. CHO-T1-SF medium lacking all proteins, casein peptone soybean flour peptone, pluronic F-68, hypoxanthine, thymidine, and methotrexate was purchased from Life Technologies (Eggenstein, Germany) as a custom made formulation. Several amino acids can be derived from animal sources, e.g. L-cysteine and L-cystine. Therefore, special care has to be taken that the medium manufacturer utilizes alternative, non-animal derived, sources for these amino acids (Jayme and Smith, 1997). HyQ-CCM5 medium (about 200 mg/l protein) was obtained from Greiner (Frickenhausen, Germany).

### *Cell Culture*

The anchorage-dependent cell lines CHO DUKXB11, CHO-A11-A2, CHO-A11-A27, CHO-A11-A279, and CHO-A11-A279-C7 were cultivated as described previously (Schröder and Friedl, 1997a). The suspension cell line CHO-SS3-A2 was cultivated in 250 ml spinner flasks at 37°C, 10% $CO_2$ and stirring at

30 rpm. Spinner flasks were inoculated with at least $1 \cdot 10^5$ cells/ml. The composition of the serum-free medium CHO-T1-SF is described in detail in the "Results and Discussion" section. Colony forming efficiencies at seeding densities of 23 or 230 cells/cm$^2$ were determined as described before (Schröder and Friedl, 1997b). Conditioned media for clonal growth assays were collected 3 h after seeding cells at $2 \cdot 10^4$ cells/cm$^2$ into 75 cm$^2$ T-flasks as described by Schröder and Friedl (1997a).

*Adaptation of cell lines to serum-free conditions*

All cell lines were weaned off 10% (v/v) fetal calf serum (FCS) by stepwise decreasing the serum concentration by 50% in every other passage. At the beginning of the weaning process the basal medium was Ham's F12 (Ham, 1965) for CHO DUKXB11 cells and DMEM (Dulbecco and Freeman, 1959; Smith et al., 1960) supplemented with 40 mg/l L-proline (Kao and Puck, 1967, 1968) for the recombinant cell lines. Below a serum concentration of 1.25% (v/v) the basal medium was switched to IF-medium (Friedl et al., 1989), and below 0.4% (v/v) the culture vessels were coated with 5 µg/cm$^2$ fibronectin. At a serum concentration of 0.1% (v/v) the basal medium was switched to CHO-T1-SF, which enabled us to completely eliminate serum from the medium. 0.1% (v/v) ExCyte III was included in early formulations of the serum-free medium. In later formulations it was omitted from the medium, since no growth promoting effects were observed (see “Results and Discussion”). The suspension cell line CHO-SS3-A2 was previously grown in a serum-free medium (Büntemeyer et al., 1991) and grew readily in medium CHO-T1-SF.

*Adaptation of cell lines to a protein-free medium*

To adapt dhfr$^-$ CHO cells to a protein-free medium the following modifications to medium CHO-T1-SF were introduced: fetuin and coating of the culture vessels with fibronectin were omitted without observing any detrimental effects. *holo*-transferrin was replaced with 7.5 µM tropolone (Merck, Darmstadt, Germany) as an iron chelator and 2.5 µM Fe(III) in the chemical form of ferric ammonium citrate (Fluka, Neu-Ulm, Germany; Metcalfe et al., 1994). Cells grown under these conditions were stepwise weaned off insulin. The insulin concentration was decreased to 10 ng/ml without adverse effects on cell growth. Then, the insulin concentration was decreased in tenfold steps to 100 pg/ml, and then omitted from the culture. We designate the protein-free formulation as medium CHO-T1-PF.

*Analytical Procedures*

The following methods were described previously: Collection and storage of conditioned media, the preparation of cell extracts (Schröder and Friedl, 1997a), cell counting (Warburton and James, 1993), the determination of ATIII (Schröder et al., 1996), and L-lactate dehydrogenase (L-LDH) (Vassault, 1987). The fraction of cells lysed during culture was calculated as described previously (Schröder and Friedl, 1997a).

## Results and discussion

*Adaptation of CHO DUKXB11 and recombinant cell lines to serum-free conditions*

The gradual reduction of the serum concentration increases the chances for successful adaptation of cells to a serum-free environment (Kim et al., 1999). For this reason we decided to stepwise decrease the serum concentration in every other passage by 50% to wean cells off serum. In this way the serum concentration could be easily reduced from 10% (v/v) to 1.25% (v/v). To further decrease the serum concentration it was necessary to switch to a basal medium formulation richer in nutrients. For CHO DUKXB11 cells the basal medium was originally Ham's F12, and for the recombinant cell lines DMEM supplemented with 40 mg/l L-proline. Changing the basal medium for all cell lines to IF-medium, a 1:1 mixture of Ham's F12 medium and IMDM (Friedl et al., 1989), allowed us to decrease the serum concentration to 0.4% (v/v). To decrease the serum concentration below 0.4% (v/v) a switch to our serum-free medium formulation CHO-T1-SF, which is even richer in nutrients than IF-medium, was necessary. In addition, the culture dishes were coated with 5 µg/cm$^2$ affinity-purified fibronectin. Initially, HEPES was used as buffer. Cells grown in this formulation usually reached maximum densities around $8 \cdot 10^4$ cells/cm$^2$. Replacing HEPES with a sodium carbonate buffer resulted in much higher cell densities in stationary phase, usually around $4 \cdot 10^5$ cells/cm$^2$. In sodium carbonate buffered CHO-T1-SF medium serum was reduced to 0.1% (v/v), and then completely eliminated from the culture.

The final composition of the serum-free medium CHO-T1-SF is listed in Table 1. Compared to other basal media (Morton, 1970; Rutzky and Pumper, 1974) the medium is very rich in metabolites, e.g. D-glucose, L-glutamine, and other L-amino acids. The protein content totals to 20 mg/l, which is significantly lower than that of other serum-free media, e.g. EX-CELL 301 (100 mg/l; Wyatt, 1994) or

UC203 (1 g/l; Ii et al., 1995). Only one component, casein peptone soybean flour peptone broth, is undefined, but aproteinous and an additional source of amino acids. Of animal origin were fetuin, insulin, *holo*-transferrin, and casein peptone soybean flour peptone (for details see Materials and Methods). However, bovine insulin can easily be replaced by recombinant human insulin (Keen and Rapson, 1995). The sodium bicarbonate content of the medium requires buffering with 10% $CO_2$ in air (Esser, 1988).

*Influence of ExCyte III on clonal growth*

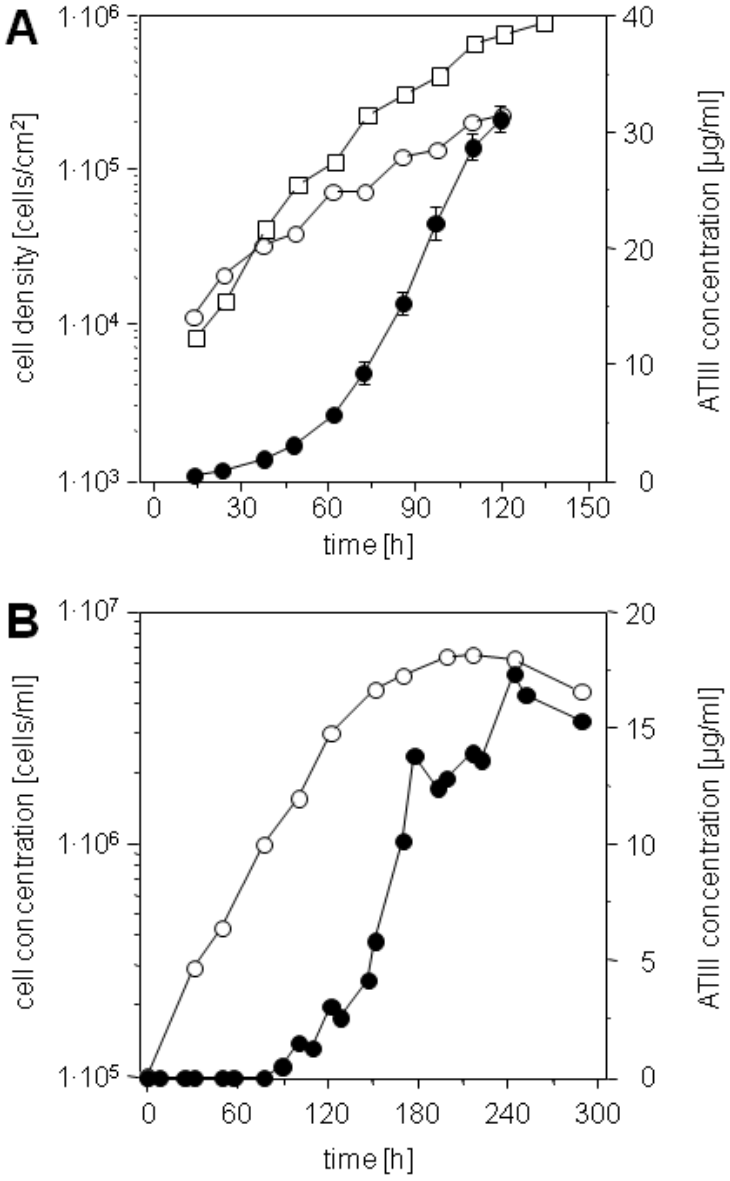
Addition of cholesterol sources to serum-free media enhances growth of several cell lines (Hink, 1991). ExCyte III is an optimal source for cholesterol and other lipids in a lipoprotein-bound manner (Büntemeyer et al., 1994). Therefore, we initially included 0.1% (w/v) ExCyte III in our serum-free medium. However, no growth was observed in this formulation when cells were seeded at very low cell densities (230 cells/$cm^2$). In the course of determining the cause for this observation, we noted an inhibitory effect of ExCyte III on growth at low densities. To examine the influence of ExCyte III on growth of our CHO cell lines at low densities in more detail, we determined the colony forming efficiencies in the presence and absence of 0.1% (w/v) ExCyte III at two different seeding densities. No cell line formed colonies when seeded at 23 cells/$cm^2$ or 230 cells/$cm^2$ in medium CHO-T1-SF + 0.1% (v/v) ExCyte III (data not shown). However, all cell lines formed colonies when plated at similar cell densities into CHO-T1-SF + 0.1% (v/v) ExCyte III conditioned for 3 h by the various cell lines as described in “Materials and Methods” (Table 2). CHO-T1-SF medium lacking ExCyte III also supported growth at these low seeding densities (Table 2). We conclude that ExCyte III inhibits growth of CHO DUKXB11 cells and recombinant CHO cells derived from CHO DUKXB11 cells at low densities. However, no inhibitory influence on cell growth at higher cell inocula, e. g. $2 \cdot 10^4$ cells/$cm^2$ (Fig. 1) was observed. A long-term requirement for ExCyte III, e.g. for a period of 20 or more passages, was not observed for any one of the cell lines used in this study (data not shown). In addition, the toxic effect of ExCyte III was abolished when conditioned medium was used. These observations indicate that the action of cellular metabolism on components in ExCyte III protects the cells from the toxic effects of ExCyte III on clonal growth of CHO cells. Another possibility is that compounds released from the cells during the conditioning process counter and outweigh the toxic effects of ExCyte III on cell growth. In accordance with our results, Büntemeyer et al. (1994) reported an

inhibitory effect of ExCyte III on growth of hybridoma cells. In contrast, cholesterol was required for long-term multiplication of SF-9 cells under serum-free conditions (Hink, 1991). The differences between the various cell lines may be due to different cholesterol requirements, endogenous synthesis rates, and cholesterol uptake rates (Chen and Kandutsch, 1983; Hewlett, 1991; Schmid, 1991).

*Colony forming efficiencies in medium CHO-T1-SF*

The colony forming efficiency in medium CHO-T1-SF after seeding at 23 cells/cm$^2$ was between 40-75% for most cell lines (Table 2). These values show no statistically significant differences to the colony forming efficiency seen in the presence of 10% (v/v) FCS (Gasser et al., 1985). When conditioned CHO-T1-SF medium plus 0.1% (w/v) ExCyte III was used, slightly lower colony forming efficiencies were observed than with fresh CHO-T1-SF medium (Table 2). Thus, the toxic effects of ExCyte III on growth at low densities are not completely reversed by the use of conditioned medium. Alternatively, metabolic waste products may have already accumulated to levels in conditioned medium at which they exert an inhibitory effect on growth at low densities and therefore counteract the protective function of conditioning against ExCyte III. However, the colony forming efficieny in CHO-T1-SF medium was lower than in the commercial serum-free medium formulation HyQ-CCM5 for cell lines CHO DUKXB1, CHO-A11-A2, CHO-A11-A27, and CHO-A11-A279 (Table 2). The higher protein content of HyQ-CCM5 medium, 200 mg/l versus 20 mg/l for CHO-T1-SF medium, may make this medium especially suited for growth and survival of cells at low seeding densities. In addition, the colony forming efficiency for cell line CHO-A11-A2 was significantly lower than for the other cell lines in medium CHO-T1-SF (Table 2). This may be due to additional nutritional requirements of this particular cell line, or incomplete adaptation of these cells to CHO-T1-SF medium.

Fig. 1. Schröder, Matischak, and Friedl




*Phenotype of CHO DUKXB11 cells adapted to serum-free conditions*

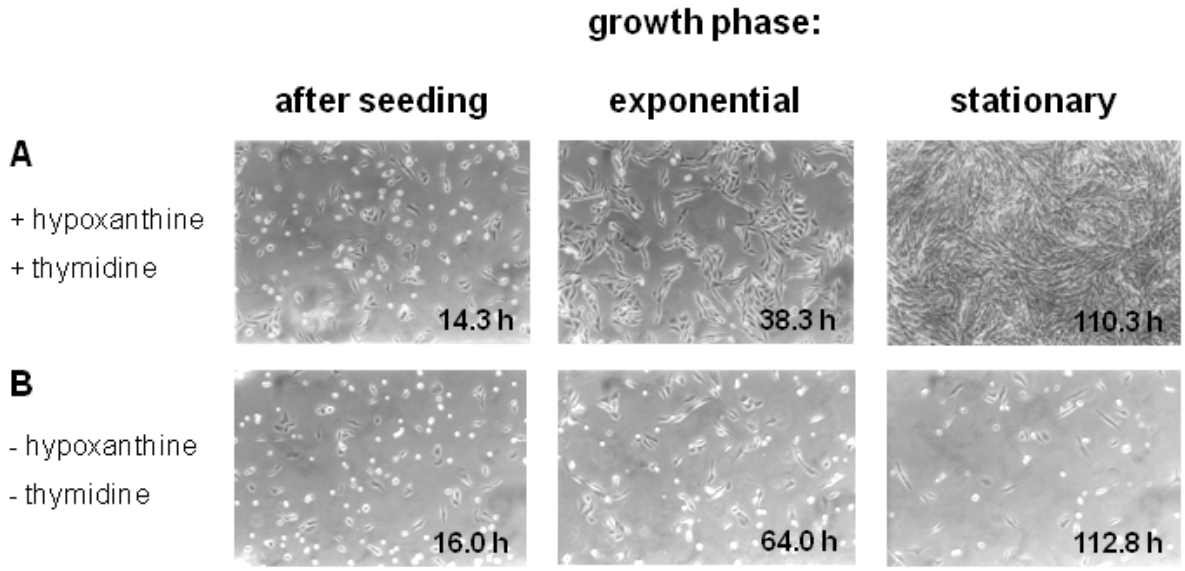
One important feature of CHO DUKXB11 cells is that they lack DHFR. However, in CHO DUKXB11 cells only one allele carries a deletion, whereas the other carries a point mutation (Gandor et al., 1995). This allele can revert, and result in overgrowth of the culture by $dhfr^+$ cells during adaptation to serum-free conditions (Gandor et al., 1995; Sinacore et al., 1996). For this reason we tested if reversion to $dhfr^+$ and overgrowth of $dhfr^-$ cells by $dhfr^+$ cells occurred during the adaptation process to serum-free conditions. CHO DUKXB11 cells adapted to serum-free conditions were seeded at $2 \cdot 10^4$ cells/$cm^2$ into a 25 $cm^2$ T-flask containing CHO-T1-SF medium lacking hypoxanthine and thymidine. Under these conditions only $dhfr^+$ cells grow, since hypoxanthine and thymidine are essential nutrients for $dhfr^-$ cells (Kaufman, 1990). After growth for ten days at 37°C, 10% $CO_2$, and medium replacement every other day no viable cells were visible in the flask and no colonies had formed (Fig. 2). As our CHO-T1-SF medium supports growth of CHO DUKXB11 cells at seeding densities as low as 23 cells/$cm^2$, we conclude that overgrowth of the initial culture by $dhfr^+$ cells did not happen.

*Growth characteristics in serum-free medium CHO-T1-SF*

Next we were interested in how well our new serum-free medium formulation supports growth of CHO cell lines at higher cell densities. First, we decided to determine generation times for CHO cell lines in the serum-free medium. To exclude inhibitory effects of substrate limitation on cell growth the generation time in the exponential growth phase was determined. Therefore, growth curves for all cell lines were recorded after seeding each cell line with a density of $2 \cdot 10^4$ cells/$cm^2$ into ten 8.7 $cm^2$ dishes. Every 12 h one dish was sacrificed for collection of conditioned media and cell counting. The culture medium in the remaining dishes was replaced every 24 h. The generation time in the exponential growth phase (Table 3) was calculated by linear regression from the slope of the first four to six data points of the growth curve (Fig. 1). Most anchorage-dependent cell lines had generation times of ≈12 h in the exponential growth phase. This is the optimum for rodent cells *in vitro* (Parchment and Natarajan, 1992). For other serum-free media generation times of ≈24 h were reported (Gasser et al., 1985; Lee et al., 1996; Renner et al., 1995; Sharif and Stevenson, 1993; Sinacore et al., 1996). Only for medium UC203 generation times similar to those observed in medium CHO-T1-SF were reported (Ii et al., 1995). Furthermore, CHO DUKXB11 cells and the recombinant cell lines CHO-A11-A2, CHO-A11-A27, and CHO-A11-A279 reached densities around

$4 \cdot 10^5$ cells/cm$^2$. Thus, CHO-T1-SF medium supports growth of these cell lines to high densities. We observed no limitation for the number of doublings (≥ about 100 doublings) that all cell lines could be maintained in CHO-T1-SF medium. This suggests that dilution of essential nutrients below critical concentrations through several passages did not occur in our medium formulation.

Fig. 2. Schröder, Matischak, and Friedl



The recombinant cell line CHO-A11-A279-C7 displayed an elevated generation time and reached maximum densities of 1-2·$10^5$ cells/$cm^2$, which was consistently less than for the other cell lines used in this study. ATIII accumulates in an immature and aggregated state in the endoplasmic reticulum of these cells (Schröder and Friedl, 1997a, 2002), which most likely activates an intracellular stress response to the aggregated protein, resulting in inhibition of translation, inhibition of cell cycle progression and apoptosis (Liu and Kaufman, 2003). Utilization of D-glucose and production of L-lactate by all four recombinant cell lines was very similar (Schröder and Friedl, 1997a), and only CHO-A11-A279-C7 cells showed an increase in utilization of L-glutamine and production of ammonia. This increase in ammonia production was offset by the lower cell density at which CHO-A11-A279-C7 cells enter stationary phase, resulting in similar ammonia concentrations for all cell lines (data not shown).

To determine the generation time of suspension cells spinner flasks were inoculated with 1·$10^5$ cells/ml of the suspension cell line CHO-SS3-A2 and incubated with stirring at 30 rpm. At the times indicated in Fig. 1 samples for cell counting were withdrawn. We observed a generation time of ≈25 h in CHO-T1-SF medium, a value that is comparable to other results published for CHO cells grown in suspension (Gasser et al., 1985; Lee et al., 1996; Renner et al., 1995; Sharif and Stevenson, 1993; Sinacore et al., 1996).

Exponential growth of all cell lines commenced directly after seeding with no lag-phase (Fig. 1). The presence of a lag-phase can indicate the requirement of medium conditioning for cell growth. The absence of a lag-phase is consistent with growth at low densities of all cell lines in medium CHO-T1-SF and demonstrates the high degree of adaptation of these cell lines to our medium formulation. In contrast, in medium UC203, in which CHO cells also had a generation time of ≈12 h, a lag-phase of about 48 h was observed (Ii et al., 1995).

*Cell lysis under serum-free conditions*

Teige et al. (1994) reported that cell lysis can be a major problem when ATIII is produced in serum-free media. This prompted us to investigate the degree of cell lysis in medium CHO-T1-SF after inoculation at higher cell densities (2·$10^4$ cells/$cm^2$). The extent of cell lysis during the cultivation of mammalian cells can be determined by measuring the activity of L-LDH in conditioned media (Bour et al., 1988; Goergen et al.,

1993; Jauregul et al., 1981; Legrand et al., 1992; Racher et al., 1990). However, we were not able to detect any L-LDH activity in media conditioned by high-density cultures of any one of the anchorage-dependent cell lines used in this study. In contrast, L-LDH activity was readily detected in cell extracts of all cell lines. The intracellular L-LDH content of $1 \cdot 10^6$ cells was 0.20 ± 0.01 units for CHO DUKXB11 cells, 0.92 ± 0.02 units for CHO-A11-A2 cells, 1.02 ± 0.05 units for CHO-A11-A27 cells, 1.05 units for CHO-A11-A279 cells, and 0.34 ± 0.02 units for CHO-A11-A279-C7 cells. The relative amounts of intracellular L-LDH corresponded to previously published data (Schröder and Friedl, 1997a). The detection limit of our L-LDH assay is ≈0.0025 units/ml in conditioned medium, and from this we estimate that during a 24 h conditioning phase less than 1% of the cells, if any, lysed. These results are in sharp contrast to those reported by Teige et al. (1994). Teige et al. (1994) used CHO cells that were not weaned off serum for their serum-free culture, whereas in our study a careful adaptation of our CHO cell lines to serum-free conditions and a medium optimization took place. These different approaches to the serum-free cultivation process can explain the dramatic differences in the sensitivity of CHO cells to serum-free conditions observed in both studies.

*Production of ATIII under serum-free conditions*

Next, we were interested in how well medium CHO-T1-SF supports the production of a recombinant protein. Conditioned media collected during the recording of the growth curves were assayed for ATIII by an enzyme-linked immunosorbent assay (ELISA) (Schröder et al., 1996) and the cumulated ATIII concentration plotted against the cultivation time (Fig. 1). ATIII accumulated to ≈30 mg/l in anchorage-dependent cultures of CHO-A11-A279-C7 cells (Fig. 1A) and to ≈15 mg/l in suspension cultures of CHO-SS3-A2 cells (Fig. 1B). Thus, medium CHO-T1-SF is suitable for the production of recombinant proteins either with anchorage-dependent or suspension cells. ATIII secreted from all recombinant cell lines was biologically active as judged from its specific progressive activity and specific heparin cofactor activity (Schröder et al., 1997a). A significant effect of ExCyte III on ATIII-secretion or activity was not observed (data not shown). To compare the suitability of CHO-T1-SF medium for production of ATIII we determined specific ATIII secretion rates for various anchorage-dependent recombinant cell lines producing human ATIII in medium CHO-T1-SF and medium CHO-T1-SF supplemented with 10% (v/v) or 1% (v/v) FCS.

Specific ATIII secretion rates were 2.5 to 3.8-fold higher in the presence of FCS (Table 4) when compared to previously published values for the serum-free medium (Schröder and Friedl, 1997a) and correspond to specific ATIII secretion rates published previously for these cell lines (Zettlmeissl et al., 1987). Furthermore, specific ATIII secretion rates and accumulation of ATIII under serum-free conditions were comparable to previously published data for other recombinant proteins produced in CHO cells under serum-free conditions, e.g. human macrophage colony stimulating factor (Sinacore et al., 1996), human clotting factor IX (Sinacore et al., 1996), γ-interferon (Gu et al., 1997; Xie et al., 1997), and the humanized antibody CAMPATH-1H (Keen and Rapson, 1995). However, the increase in productivity under serum-supplemented conditions was offset by lower cell densities of confluent cultures in the presence of FCS (usually around $4 \cdot 10^4$ cells/cm$^2$). In addition, in practice more demanding and cost-intensive purification strategies for ATIII from serum-supplemented cultures would be necessary to isolate ATIII from serum-supplemented cultures. This is, for example, illustrated by the observation that ATIII secreted from CHO-A11-A2 cells in the presence of 10% (v/v) FCS did not accumulate over background levels over 24 h (Table 4). In summary, we conclude that production of ATIII in the serum-free medium CHO-T1-SF is superior to production of ATIII in serum-supplemented cultures.

*Protein-free medium formulation*

Finally, we wanted to know how easily a protein-free medium formulation can be developed on the basis of the serum-free formulation. First, we tried to omit the protein components from the medium formulation. Omission of fetuin and coating of the tissue culture dishes with 5 µg/cm$^2$ fibronectin did not interfere with growth of any cell line. However, the cells were sensitive to removal of *holo*-transferrin and insulin. Iron saturated heavy ion chelators, such as tropolone, are an excellent replacement for *holo*-transferrin (Metcalfe et al., 1994). Addition of 2.5 µM Fe(III) in the form of ferric ammonium citrate, and 7.5 µM tropolone to our medium formulation supported long-term growth of CHO cell lines in the absence of *holo*-transferrin (data not shown). To eliminate insulin from the culture process we decided to wean the cells off insulin in a similar way as performed for weaning the cells off serum. The insulin concentration was decreased from 10 µg/ml to 10 ng/ml, then to 1 ng/ml, and to 0.1 ng/ml, and finally omitted from the culture process. Cell lines CHO DUKXB11, CHO-A11-A2, CHO-A11-A27, and CHO-A11-A279 were

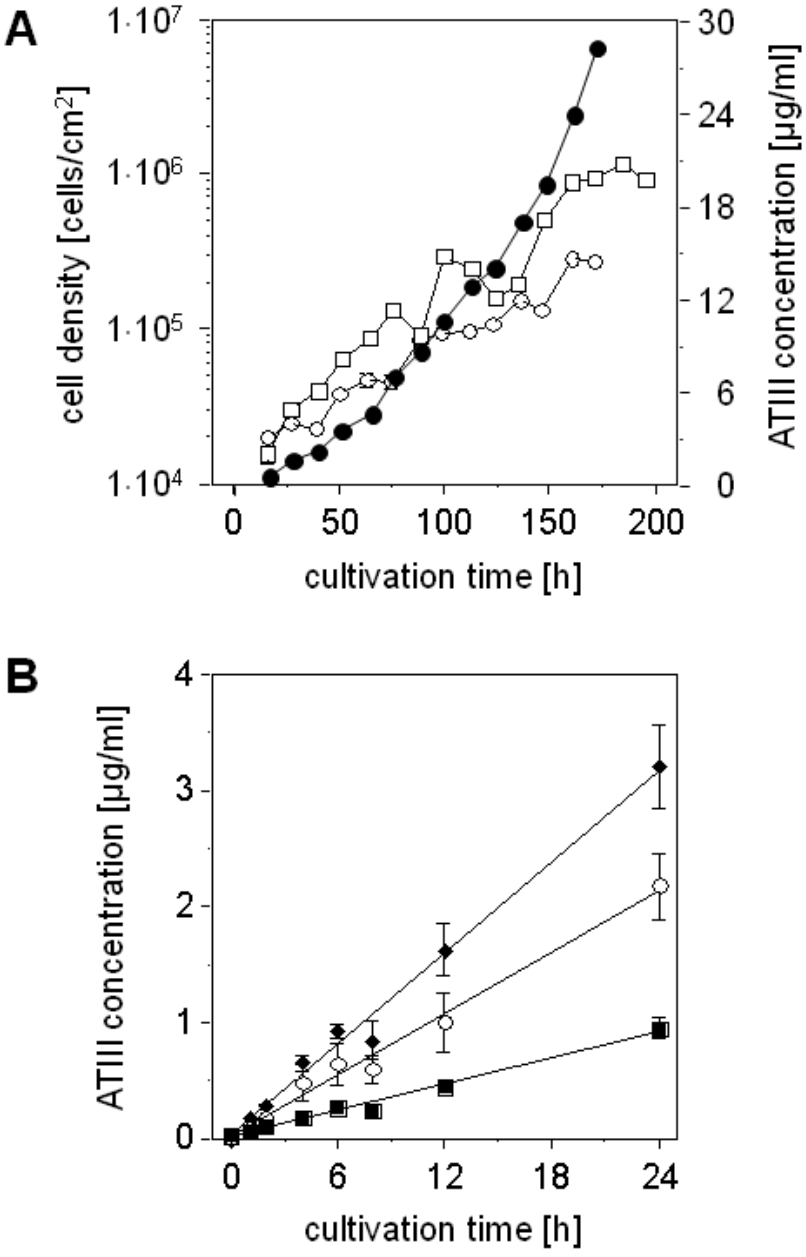
successfully adapted to a protein-free medium formulation and could be maintained in the protein-free medium for at least 50 doublings. However, we were not able to adapt cell line CHO-A11-A279-C7 to insulin-free conditions, which may be due to an intrinsic stressed state of this cell line (see above for discussion). Further engineering of mammalian cell lines, especially by increasing the capacity of the secretory pathway for the production of recombinant secretory proteins, and uncoupling of cell cycle control from control by stress responses initiated in the secretory pathway (Calfon et al., 2002; Reimold et al., 2001; Schröder et al., 2000), may be necessary to develop protein-free culture conditions for highly overexpressing cell lines. In summary, cells grown in our serum-free medium formulation can rapidly and easily be adapted to a completely protein-free medium formulation.

*Growth characteristics and ATIII secretion in protein-free medium*

To determine how well the various cell lines were adapted to the protein-free medium we measured growth characteristics and ATIII secretion. Experiments similar to those described above for the serum-free medium formulation revealed that generation times are significantly increased in the protein-free formulation for cell lines CHO DUKXB11, CHO-A11-A2, and CHO-A11-A279 when compared to the serum-free formulation (Table 5). However, they are comparable with generation times reported for CHO SSF3 cells producing urokinase-type plasminogen activator (about 27 h, Zang et al., 1995) or a humanized immunoglobin G kappa light chain (about 15 h, Zang et al., 1995). The increased generation times in the protein-free medium are probably due to a lack of insulin and indicate a partial block in progression through the cell cycle, presumably a prolongation of the G1-phase. However, all cell lines initiated growth after seeding with no obvious lag-phase (Fig. 3A), indicating that conditioning of the medium was not required for growth. In addition, the protein-free medium routinely supported growth of cultures to densities of $1 \cdot 10^5$ to $2 \cdot 10^5$ cells/cm$^2$ in stationary phase (Fig. 3A and data not shown). Last, the protein-free medium had no adverse effects on ATIII-secretion. Accumulation of ATIII in protein-free medium conditioned by stationary cultures (Fig. 3B) was indistinguishable from serum-free conditions (Fig. 3 in Schröder and Friedl, 1997a). Further, specific ATIII secretion rates for CHO-A11-A2, CHO-A11-A27, and CHO-A11-A279 cells in protein-free medium were comparable to serum-supplemented cultures (Table 4) and 2.5 to 3.5-fold enhanced when compared to previously published values for the serum-free

formulation (Schröder and Friedl, 1997a). However, this increase in cellular productivity in the protein-free medium was offset by lower cell densities in stationary phase, resulting in a similar accumulation of ATIII under both conditions. Finally, ATIII produced by CHO-A11-A279 cells accumulated to about 30 mg/l in stationary phase (Fig. 3A). This is comparable to the accumulation of urokinase-type plasminogen activator secreted by CHO SSF3 cells in a protein-free environment, which reached 60-70 mg/l in stationary phase (Zang et al., 1995). We conclude that the protein-free formulation is suitable for routine maintenance of CHO cell lines, growth to high cell densities, and the production of recombinant proteins.

Fig. 3. Schröder, Matischak, and Friedl



*Conclusions*

In this report we present serum- and protein-free media for dhfr$^-$ CHO DUKXB11 cells and recombinant cell lines expressing ATIII derived from CHO DUKXB11 cells. The serum-free medium has a low protein content compared to other serum-free media for CHO cells and supports growth of the cell lines investigated in this study at low and high seeding densities. Only minor changes in the medium formulation were necessary for its use in the cultivation of anchorage-dependent and suspension cells. Adaptation of cells grown in the serum-free formulation to a protein-free medium was easy and straightforward. This did also result in elimination of most animal-derived components from the medium. Further optimization of this medium formulation should try to replace the peptone used in this study with a defined set of amino acids and peptides. In addition, the combination of having a dhfr$^-$ cell line adapted to serum- and even protein-free conditions at hand, may provide a reference point for the development of recombinant CHO cell lines that either grow in suspension or are anchorage-dependent. The ease with which the suspension cell line CHO-SS3-A2, which was maintained in another serum-free medium prior to culture in CHO-T1-SF medium (Büntemeyer et al., 1991), was adapted to CHO-T1-SF medium suggests that our medium formulation may be useful in the cultivation of other CHO cell lines.

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## References

Asakura, E., Yamauchi, T., Amatsuji, Y., Uno, S., Furuta, R., Tsujikawa, M., Tanabe, T., and Yokoyama, K. (1992) Production of human antithrombin-III in a serum-free culture of CHO cells. In: Murakami, H., Shirahata, S., and Tochibara, H. (Eds.), Animal Cell Technology: Basic & Applied Aspects. Kluwer Academic Publishers, Dordrecht, pp. 345-351.

Barnes, D., and Sato, G. (1980) Methods for growth of cultured cells in serum-free medium. Anal. Biochem. 102, 255-270.

Blüml, G., Reiter, M., Zach, N., Gaida, T., Schmatz, C., Assadian, A., Strutzenberger, K., and Katinger, H. (1994) Protein free culture of r-CHO and hybridoma cells on the macroporous polypore® carrier. In: Spier, R.E., Griffiths, J.B., and Berthold, W. (Eds.), Animal Cell Technology: Products of Today, Prospects for Tomorrow. Butterworth-Heinemann, Oxford, pp. 267-269.

Bour, J.M., Maugras, M., Capiaumont, J., Dousset, B., Straczek, J., Gelot, M.A., and Nabet, P. (1988) Marqueurs biochimiques de la prolifération et de la mort des hybridomes en culture. Bio-Sciences 7, 35-40.

Büntemeyer, H., Lütkemeyer, D., and Lehmann, J. (1991) Optimization of serum-free fermentation processes for antibody production. Cytotechnology 5, 57-67.

Büntemeyer, H., Burg, M., and Lehmann, J. (1994) Cholesterol: An important additive for serum-free media? In: Spier, R.E., Griffiths, J.B., and Berthold, W. (Eds.), Animal Cell Technology: Products of Today, Prospects for Tomorrow. Butterworth-Heinemann, Oxford, pp. 124-126.

Calfon, M., Zeng, H., Urano, F., Till, J.H., Hubbard, S.R., Harding, H.P., Clark, S.G., and Ron, D. (2002) IRE1 couples endoplasmic reticulum load to secretory capacity by processing the *XBP-1* mRNA. Nature 415, 92-96.

Chen, H.W., and Kandutsch, A.A. (1983) Cholesterol requirements for cell growth: Endogenous synthesis versus exogenous sources. In: Waymouth, C. (ed.), The Growth Requirements of Vertebrate Cells in Vitro. Cambridge University Press, Cambridge, pp. 327-342.

Crouse, G.F., McEwan, R.N., and Pearson, M.L. (1983) Expression and amplification of engineered mouse dihydrofolate reductase minigenes. Mol. Cell. Biol. 3, 257-266.

Dulbecco, R., and Freeman, G. (1959) Plaque production by the polyoma virus. Virology 8, 396-397.

Esser, P. (1988) pH and pressure in closed tissue culture vessels. Nunc. Bull. 5, 1-3.

Friedl, P., Tatje, D., and Czapla, R. (1989) An optimized culture medium for human vascular endothelial cells from umbilical cord veins. Cytotechnology 2, 171-179.

Gandor, C., Leist, C., Fiechter, A., and Asselbergs, F.A.M. (1995) Amplification and expression of recombinant genes in serum-independent Chinese hamster ovary cells. FEBS Lett. 377, 290-294.

Gasser, F., Mulsant, P., and Gillois, M. (1985) Long-term multiplication of the Chinese hamster ovary (CHO) cell line in a serum-free medium. In Vitro Cell. Dev. Biol. 21, 588-592.

Goergen, J.L., Marc, A., and Engasser, J.M. (1993) Determination of cell lysis and death kinetics in continuous hybridoma cultures from the measurement of lactate dehydrogenase release. Cytotechnology 11, 189-195.

Gu, X., Xie, L., Harmon, B.J., and Wang, D.I.C. (1997) Influence of Primatone RL supplementation on sialylation of recombinant human interferon-$\gamma$ produced by Chinese hamster ovary cell culture using serum-free media. Biotechnol. Bioeng. 56, 353-360.

Ham, R.G. (1965) Clonal growth of mammalian cells in a chemically defined, synthetic medium. Proc. Natl. Acad. Sci. USA 53, 288-293.

Hamilton, W.G., and Ham, R.G. (1977) Clonal growth of Chinese hamster cell lines in protein-free media. In Vitro 13, 537-547.

Hata, J.I., Tamura, T., Yokoshima, S., Yamashita, S., Kabeno, S., Matsumoto, K., and Onodera, K. (1992) Chemically defined medium for the production of biologically active substances of CHO cells. Cytotechnology 10, 9-14.

Hewlett, G. (1991) Lipoprotein requirements of cultured mammalian cells. In: Spier, R.E., Griffiths, J.B., and Meignier, B. (Eds.), Production of Biologicals from Animal Cells in Culture. Butterworth-Heinemann, Oxford, pp. 67-69.

Hink, W.F. (1991) A serum-free medium for the culture of insect cells and production of recombinant proteins. In Vitro Cell. Dev. Biol. 27A, 397-401.

Ii, T., Murakami, M., Mizuguchi, M., Matsumoto, K., and Onodera, K. (1995) Production of recombinant human thyroid peroxidase in Chinese hamster ovary cells. In: Beuvery, E.C., Griffiths, J.B., and Zeijlemaker, W.P. (Eds.), Animal Cell Technology: Developments Towards the 21st Century. Kluwer Academic Publishers, Dordrecht, pp. 385-389.

Jauregul, H.O., Hayner, N.T., Driscoll, J.L., Williams-Holland, R., Lipsky, M.H., and Galletti, P.M. (1981) Trypan blue dye uptake and lactate dehydrogenase in adult rat hepatocytes - freshly isolated cells, cell suspensions, and primary monolayer cultures. In Vitro 17, 1100-1110.

Jayme, D.W., and Smith, S.R. (1997) Options to eliminate animal-derived components of cell culture media. In: Kitagawa, Y., Matsuda, T., and Iijima, S. (Eds.), Animal Cell Technology: Basic & Applied Aspects, Vol. 10, Kluwer Academic Publishers, Dordrecht, pp.199-203.

Kane, S.E. (1991) High-level expression of foreign genes in mammalian cells. In: Setlow, J.K. (Ed.), Genetic Engineering, Vol. 13, Plenum Press, New York, pp. 167-182.

Kao, F.-T., and Puck, T.T. (1967) Genetics of somatic mammalian cells. IV. Properties of Chinese hamster cell mutants with respect to the requirement for proline. Genetics 55, 513-524.

Kao, F.-T., and Puck, T.T. (1968) Genetics of somatic mammalian cells. VII. Induction and isolation of nutritional mutants in Chinese hamster cells. Proc. Natl. Acad. Sci. USA 60, 1275-1281.

Kaufman, R.J. (1990) Selection and coamplification of heterologous genes in mammalian cells. Methods Enzymol. 185, 537-566.

Kaufman, R.J. (1993) Amplification and expression of transfected genes in mammalian cells. In: Kellems, R.E. (Ed.), Gene Amplification in Mammalian Cells, Marcel Dekker, New York, pp. 315-343.

Keen, M.J., and Rapson, N.T. (1995) Development of a serum-free culture medium for the large scale production of recombinant protein from a Chinese hamster ovary cell line. Cytotechnology 17, 153-163.

Kellems, R.E. (1991) Gene amplification in mammalian cells: strategies for protein production. Curr. Opin. Biotechnol. 2, 723-729.

Kim, E.J., Kim, N.S., and Lee, G.M. (1998) Development of a serum-free medium for the production of humanized antibody from Chinese hamster ovary cells using a statistical design. In Vitro Cell. Dev. Biol. - Animal 34, 757-761.

Kim, E.J., Kim, N.S., and Lee, G.M. (1999) Development of a serum-free medium for dihydrofolate reductase-deficient Chinese hamster ovary cells (DG44) using a statistical design: beneficial effect of weaning of cells. In Vitro Cell. Dev. Biol. - Animal 35, 178-182.

Kimberlin, R.H. (1991) An overview of bovine spongiform encephalopathy. Dev. Biol. Stand. 75, 75-82.

Kitano, K. (1994) Improvement of cell lines for large-scale mammalian cell culturing. In: Spier, R.E., Griffiths, J.B., and Berthold, W. (Eds.), Animal Cell Technology: Products of Today, Prospects for Tomorrow. Butterworth-Heinemann, Oxford, pp. 35-40.

Lee, G.M., Kim, E.J., Kim, N.S., Yoon, S.K., Ahn, Y.H., and Song, J.Y. (1999) Development of a serum-free medium for the production of erythropoietin by suspension culture of recombinant Chinese hamster ovary cells using a statistical design. J. Biotechnol. 69, 85-93.

Lee, K., Sburlati, A., Renner, W.A., and Bailey, J.E. (1996) Deregulated expression of cloned transcription factor E2F-1 in Chinese hamster ovary cells shifts protein patterns and activates growth in protein-free medium. Biotechnol. Bioeng. 50, 273-279.

Legrand, C., Bour, J.M., Jacob, C., Capiaumont, J., Martial, A., Marc, A., Wudtke, M., Kretzmer, G., Demangel, C., Duval, D., and Hache, J. (1992) Lactate dehydrogenase (LDH) activity of the number of dead cells in the medium of cultured eukaryotic cells as marker. J. Biotechnol. 25, 231-243.

Liu, C.Y., and Kaufman, R.J. (2003) The unfolded protein response. J. Cell. Sci. 116, 1861-1862.

Maurer, H.R. (1986) Towards chemically-defined, serum-free media for mammalian cell culture. In: Freshney, R.I. (ed.), Animal Cell Culture - A Practical Approach, IRL Press. Oxford, pp. 13-31.

Metcalfe, H., Field, R.P., and Froud, S.J. (1994) The use of 2-hydroxy-2,4,6-cycloheptarin-1-one (tropolone) as a replacement for transferrin. In: Spier, R.E., Griffiths, J.B., and Berthold, W. (Eds.), Animal Cell Technology: Products of Today, Prospects for Tomorrow. Butterworth-Heinemann, Oxford, pp. 88-90.

Minor, P.D. (1994) Ensuring safety and consistency in cell culture production processes: viral screening and inactivation. Trends Biotechnol. 12, 257-261.

Mizuguchi, M., Matsumoto, K., and Onodera, K. (1993) Production of the human growth hormone in serum-free UC203 medium. In: Kaminogawa, S., Ametani, A., and Hachimura, S. (Eds.), Animal Cell Technology: Basic & Applied Aspects, Vol. 5, Kluwer Academic Publishers, Dordrecht, pp. 57-61.

Morris, C.B., and Warburton, S. (1993) Serum and serum-free. Serum - screening and selection. In: Doyle, A., Griffiths, J.B., and Newell, D.G. (Eds.), Cell & Tissue Culture: Laboratory Procedures, John Wiley & Sons, Chicester, pp. 2B:1.1.-2B:1.5.

Morton, H.J. (1970) A survey of commercially available tissue culture media. In Vitro 6, 89-108.

Parchment, R.E., and Natarajan, K. (1992) A free-radical hypothesis for the instability and evolution of genotype and phenotype *in vitro*. Cytotechnology 10, 93-124.

Racher, A.J., Looby, D., and Griffiths, J.B. (1990) Use of lactate dehydrogenase release to assess changes in culture viability. Cytotechnology 3, 301-307.

Reimold, A.M., Iwakoshi, N.N., Manis, J., Vallabhajosyula, P., Szomolanyl-Tsuda, E., Gravallese, E.M., Friend, D., Grusby, M.J., Alt, F., and Glimcher, L.H. 2001. Plasma cell differentiation requires the transcription factor XBP-1. Nature 412, 300-307.

Renner, W.A., Lee, K.H., Hatzimanikatis, V., Bailey, J.E., and Eppenberger, H.M. (1995) Recombinant cyclin E expression activates proliferation of Chinese hamster ovary (CHO) cells in protein-free medium. Biotechnol. Bioeng. 47, 476-482.

Rutzky, L.P., and Pumper, R.W. (1974) Supplement to a survey of commercially available tissue culture media (1970). In Vitro 9, 468-469.

Sato, G.H. (1975) The role of serum in cell culture. In: Litwack, G.(Ed.), Biochemical Action of Hormones, Vol. III., Academic Press, New York, pp. 391-396.

Scharfenberg, K., and Wagner, R. (1995) A reliable strategy for the achievement of cell lines growing in protein-free medium. In: Beuvery, E.C., Griffiths, J.B., and Zeijlemaker, W.P. (Eds.), Animal Cell Technology: Developments Towards the 21$^{st}$ Century. Kluwer Academic Publishers, Dordrecht, pp. 619-623.

Schmid, G. (1991) Lipid metabolism of animal cells in culture - a review. In: Spier, R.E., Griffiths, J.B., and Meignier, B. (Eds.), Production of Biologicals from Animal Cells in Culture. Butterworth-Heinemann, Oxford, pp. 61-66.

Schorn, P., Noé, W., and Berthold, W. (1994) Metabolic characteristics and specific requests of hybridoma and CHO cell lines. In: Spier, R.E., Griffiths, J.B., and Berthold, W. (Eds.), Animal Cell Technology: Products of Today, Prospects for Tomorrow. Butterworth-Heinemann, Oxford, pp. 199-201.

Schröder, M., and Friedl, P. (1997a) Overexpression of recombinant human antithrombin III in Chinese hamster ovary cells results in malformation and decreased secretion of the recombinant protein. Biotechnol. Bioeng. 53, 547-559.

Schröder, M., and Friedl, P. (1997b) A protein-free solution as replacement for serum in trypsinization protocols for anchorage-dependent cells. Methods Cell Sci. 19, 137-147.

Schröder, M., and Friedl P. (2002) Induction of protein aggregation in an early secretory compartment by elevation of expression level. Biotechnol. Bioeng. 78, 131-140.

Schröder, M., Kaiser, D., Schäfer, R., and Friedl, P. (1996) Indirect sandwich ELISA for human antithrombin III employing the interaction between D-biotin and streptavidin. Genet. Eng. Biotechnol. 16, 211-225.

Schröder, M., Chang, J.S., and Kaufman, R.J. (2000) The unfolded protein response represses nitrogen-starvation induced developmental differentiation in yeast. Genes Dev. 14, 2962-2975.

Sharif, N.A., and Stevenson, D.J. (1993) Chinese hamster ovary (CHO-K1) cells: Optimum culture conditions, cell-cycle events and phospholipid metabolism response to serum and calcium ionophore A23187. Med. Sci. Res. 21, 553-556.

Sinacore, N., Charlebois, T., Harrison, S., Brennan, S., Richards, T., Hamilton, M., Scott, S., Brodeur, P., Oakes, P., Leonard, M., Switzer, M., Anagnostopoulos, A., Foster, B., Harris, A., Jankowski, M., Bond, M., Martin, S., and Adamson, S.R. (1996) CHO DUKX cell lineages preadapted to growth in serum-free suspension culture enable rapid development of cell culture processes for the manufacture of recombinant proteins. Biotechnol. Bioeng. 52, 518-528.

Smith, J.D., Freeman, G., Vogt, M., and Dulbecco R. 1960. The nucleic acid of polyoma virus. Virology 12, 185-196.

Tebbe, H., Büntemeyer, H., Lütkemeyer, D., and Lehmann, J. (1995) Rapid HPLC quantification of recombinant human antithrombin III during production and purification. In: Beuvery, E.C., Griffiths, J.B., and Zeijlemaker, W.P. (Eds.), Animal Cell Technology: Developments Towards the 21st Century. Kluwer Academic Publishers, Dordrecht, pp. 455-459.

Teige, M., Weidemann, R., and Kretzmer, G. (1994) Problems with serum-free production of antithrombin III regarding proteolytic activity and product quality. J. Biotechnol. 34, 101-105.

Urlaub, G., and Chasin, L.A. (1980) Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity. Proc. Natl. Acad. Sci. USA 77, 4216-4220.

Urlaub, G., Käs, E., Carother, A.M., and Chasin, L.A. (1983) Deletion of the diploid dihydrofolate reductase locus from cultured mammalian cells. Cell 33, 405-412.

Vassault, A. (1987) Lactate dehydrogenase. UV-method with pyruvate and NADH. In: Bergmeyer, H.U., Bergmeyer, J., and Graßl, M. (Eds.), Methods of Enzymatic Analysis, Vol. III: Enzymes 1: Oxidoreductases, Transferases, 3rd edition, VCH, Weinheim, pp. 118-126.

Warburton, S., and James, R. (1993) Hemocytometer cell counts and viability studies. In: Doyle, A., Griffiths, J.B., and Newell, D.G. (Eds.), Cell & Tissue Culture: Laboratory Procedures, John Wiley & Sons, Chicester, pp. 4B:1.1-4B:1.5.

Wyatt, D.E. (1994) Evaluation of CHO cell growth in a protein-free environment. In: Spier, R.E., Griffiths, J.B., and Berthold, W. (Eds.), Animal Cell Technology: Products of Today, Prospects for Tomorrow. Butterworth-Heinemann, Oxford, pp. 137-139.

Xie, L., Nyberg, G., Gu, X., Li, H., Möllborn, F., and Wang, D.I.C. (1997) Gamma-interferon production and quality in stoichiometric fed-batch cultures of Chinese hamster ovary (CHO) cells under serum-free conditions. Biotechnol. Bioeng. 56, 577-582.

Zang, M., Trautmann, H., Gandor, C., Messi, F., Asselbergs, F., Leist, C., Fiechter, A., and Reiser, J. (1995) Production of recombinant proteins in Chinese hamster ovary cells using a protein-free cell culture medium. Bio/Technology 13, 389 - 392.

Zettlmeissl, G., Ragg, H., and Karges, H. (1987) Expression of biologically active antithrombin III in Chinese hamster ovary cells. Bio/Technology 5, 720-725.

## Figure legends

**Fig. 1. Growth and ATIII-production curves in serum-free medium CHO-T1-SF.** (A) Growth of anchorage-dependent cell lines CHO DUKXB11 (□), CHO-A11-A279-C7 (○), and production of ATIII by CHO-A11-A279-C7 cells (●). The cells were seeded at $2 \cdot 10^4$ cells/$cm^2$ into ten 8.7 $cm^2$ dishes containing serum-free medium CHO-T1-SF. Every 12 h one dish was sacrificed for collection of conditioned media and cell counting. The culture medium in the remaining dishes was replaced every 24 h. The medium for both cell lines contained 0.1% (v/v) ExCyte III. A representative of two independent experiments is shown. (B) Growth of the suspension cell line CHO-SS3-A2 (○) and ATIII production by CHO-SS3-A2 cells (●). A spinner flask was inoculated with $1 \cdot 10^5$ cells/ml and incubated at 37°C under 10% $CO_2$ in air and a stirring rate of 30 rpm. Samples for cell counting and determination of ATIII by ELISA (Schröder et al., 1996) were withdrawn at the times indicated.

**Fig. 2. Growth of dhfr$^-$ CHO DUKXB11 cells after adaptation to serum-free conditions in the presence (A) or absence (B) of hypoxanthine and thymidine.** CHO DUKXB11 cells were seeded at $2 \cdot 10^4$ cells/$cm^2$ into 25 $cm^2$ T-flasks containing either complete CHO-T1-SF medium or CHO-T1-SF medium lacking hypoxanthine and thymidine and incubated at 37°C and 10% $CO_2$ in air. The cells were photographed at the times stated in the lower right corner of each photograph. A representative photograph of cells after seeding, in the exponential growth phase, and in the stationary phase are shown.

**Fig. 3. Growth and ATIII-production curves in protein-free medium CHO-T1-PF.** (A) Growth of anchorage-dependent cell lines CHO DUKXB11 (□), CHO-A11-A279 (○), and production of ATIII by CHO-A11-A279 cells (●). Cells were seeded at $2 \cdot 10^4$ cells/$cm^2$ into ten 8.7 $cm^2$ dishes containing protein-free medium CHO-T1-PF. Every 12 h one dish was sacrificed for collection of conditioned media and cell counting. The culture medium in the remaining dishes was replaced every 24 h. The ATIII concentration in conditioned media was determined by ELISA (Schröder et al., 1996). (B) Secretion of ATIII by stationary

cultures of CHO-A11-A2 (■), CHO-A11-A27 (○), and CHO-A11-A279 cells (♦). Cells were seeded at $2 \cdot 10^4$ cells/$cm^2$ into eight 8.7 $cm^2$ dishes containing protein-free medium CHO-T1-PF, grown to confluency and one dish sacrificed at the times indicated to count cells and to collect conditioned medium.

## Tables

**Table 1. Composition of serum-free medium CHO-T1-SF.** The pH of the medium is adjusted to 7.2 - 7.4, and the medium is incubated under 10% $CO_2$ in air (Esser, 1988).

| Component | Specific concentration [mg/l] | Molar concentration [mM] |
|---|---|---|
| **Inorganic salts:** | | |
| $CaCl_2$ | 211.288 | 1.90 |
| $CuSO_4 \cdot 5H_2O$ | 0.0012 | $4.81 \cdot 10^{-6}$ |
| $FeSO_4 \cdot 7H_2O$ | 0.3336 | $1.20 \cdot 10^{-3}$ |
| $KNO_3$ | 0.0912 | $9.02 \cdot 10^{-4}$ |
| KCl | 485.44 | 6.51 |
| $MgCl_2$ | 22.888 | 0.24 |
| $MgSO_4$ | 117.204 | 0.576 |
| NaCl | 3039.6 | 52 |
| $NaHCO_3$ | 4099.2 | 48.8 |
| $Na_2HPO_4$ | 56.816 | 0.4 |
| $NaH_2PO_4 \cdot H_2O$ | 150 | 1.25 |
| $Na_2SeO_3 \cdot 5H_2O$ | 0.02601 | $9.89 \cdot 10^{-5}$ |
| $ZnSO_4 \cdot 7H_2O$ | 0.3452 | $1.20 \cdot 10^{-3}$ |

| | | |
|---|---|---|
| **Carbohydrates:** | | |
| D-glucose | 6120.8 | 34 |
| **L-amino acids:** | | |
| L-alanine | 33.56 | 0.377 |
| L-arginine·HCl | 185.2 | 0.879 |
| L-asparagine·$H_2O$ | 40.084 | 0.267 |
| L-aspartic acid | 41.32 | 0.31 |
| L-cysteine·HCl·$H_2O$ | 14.048 | 0.08 |
| L-cystine·2HCl | 109.488 | 0.349 |
| L-glutamic acid | 95.88 | 0.652 |
| L-glutamine | 1052.28 | 7.2 |
| glycine | 39 | 0.52 |
| L-histidine·HCl·$H_2O$ | 58.784 | 0.28 |
| L-isoleucine | 127.576 | 0.973 |
| L-leucine | 131.24 | 1.00 |
| L-lysine·HCl | 189.8 | 1.04 |
| L-methionine | 37.792 | 0.253 |
| L-phenylalanine | 81.184 | 0.491 |

| | | |
|---|---|---|
| L-proline | 61.8 | 0.537 |
| L-serine | 54.6 | 0.52 |
| L-threonine | 118.76 | 0.997 |
| L-tryptophan | 20.016 | 0.098 |
| L-tyrosine, disodium salt | 128.164 | 0.569 |
| L-valine | 117.48 | 1.00 |
| **Vitamins and miscellaneous compounds:** | | |
| D-biotin | 0.01852 | $7.58 \cdot 10^{-5}$ |
| DL-pantothenic acid, calcium salt | 4.992 | $1.09 \cdot 10^{-2}$ |
| choline chloride | 10.384 | $7.44 \cdot 10^{-2}$ |
| ethanolamine | 1.22 | 0.02 |
| folic acid | 5.32 | $1.21 \cdot 10^{-2}$ |
| hypoxanthine[1)] | 13.6 | 0.1 |
| *i*-inositol | 15.84 | $8.79 \cdot 10^{-2}$ |
| linoleic acid | 0.0336 | $1.20 \cdot 10^{-4}$ |
| lipoic acid | 0.084 | $4.07 \cdot 10^{-4}$ |
| methotrexate (MTX)[2)] | | $1 \cdot 10^{-4}$ - 0.1 |
| nicotinamide | 4.8148 | $3.94 \cdot 10^{-2}$ |

| | | |
|---|---|---|
| phenol red | 18.48 | $5.21 \cdot 10^{-2}$ |
| pluronic F-68[3)] | 2000 | - |
| pyridoxal·HCl | 4.8 | $2.36 \cdot 10^{-2}$ |
| pyridoxine·HCl | 0.0248 | $1.21 \cdot 10^{-4}$ |
| riboflavin | 0.4952 | $1.32 \cdot 10^{-3}$ |
| sodium pyruvate | 276 | 2.51 |
| thiamine·HCl | 4.936 | $1.46 \cdot 10^{-2}$ |
| thymidine[1)] | 3.88 | 0.016 |
| vitamin $B_{12}$ | 0.5596 | $4.13 \cdot 10^{-4}$ |
| **Peptones and proteins:** | | |
| fetuin[4)] | 10 | - |
| insulin | 5 | - |
| *holo*-transferrin | 5 | - |
| casein peptone soybean flour peptone broth[5)] | 5000 | - |

Footnotes:

1) Hypoxanthine and thymidine were only added to the medium for the cell line CHO DUKXB11.

2) MTX was added to the medium at the appropriate concentration: 0.1 µM for CHO-A11-A2 cells, 1 µM for CHO-A11-A27, 10 µM for CHO-A11-A279 and CHO-SS3-A2 cells, and 100 µM for CHO-A11-A279-C7 cells. It was omitted from the medium for CHO DUKXB11 cells.

3) Pluronic F-68 was only added to the medium for the suspension cell line CHO-SS3-A2.

4) Fetuin was only added to the medium for the anchorage-dependent cell lines.
5) A 6 x stock solution of the casein peptone soybean flour peptone broth was filter sterilized and than added to the final medium.

**Table 2. Colony formation efficiencies for anchorage-dependent CHO cell lines in the serum-free medium CHO-T1-SF.** Confluent cultures of CHO cells were trypsinized, seeded at $2 \cdot 10^4$ cells/$cm^2$ into 25 $cm^2$ T-flasks, incubated at 37°C and 10% $CO_2$ in air for two days, trypsinized, seeded at 23 cells/$cm^2$ into 8.7 $cm^2$ dishes, and incubated at 37°C and 10% $CO_2$ in air for seven days without removing the dishes from the incubator. Colonies were stained with 1% (w/v) crystal violet in 70% (v/v) ethanol and counted. The colony formation efficiency was calculated as the percentage of colonies formed per seeded cells.

| | **Colony formation efficiency [%]** | | |
|---|---|---|---|
| **Cell Line** | **conditioned CHO-T1-SF + 0.1% (v/v) ExCyte III** | **CHO-T1-SF** | **HyQ-CCM5** |
| CHO DUKXB11 | 39 ± 3 | 47 ± 3 | 100 ± 36 |
| CHO-A11-A2 | 15 ± 4 | 19 ± 6 | 99 ± 10 |
| CHO-A11-A27 | 52 ± 8 | 74 ± 12 | 81 ± 11 |
| CHO-A11-A279 | 49 ± 7 | 45 ± 6 | 100 ± 20 |
| CHO-A11-A279-C7 | 65 ± 9 | 73 ± 10 | 43 ± 12 |

**Table 3. Generation times in serum-free medium CHO-T1-SF.** The generation time in the exponential growth phase was calculated by linear regression from the slope of growth curves as shown in Fig. 1. Only the first four to six data points were used for regression analysis.

| Cell line | Generation time [h] |
|---|---|
| CHO DUKXB11 | 10.4 ± 0.2 |
| CHO-A11-A2 | 11.6 ± 1.2 |
| CHO-A11-A27 | 13.3 ± 1.7 |
| CHO-A11-A279 | 12.6 ± 0.7 |
| CHO-A11-A279-C7 | 19.2 ± 2.0 |
| CHO-SS3-A2 | 25.4 ± 1.0 |

**Table 4. Specific ATIII secretion rates in serum- and protein-free media and serum supplemented media.** Specific ATIII secretion rates for confluent cultures of CHO-A11-A2, CHO-A11-A27, CHO-A11-A279, and CHO-A11-A279-C7 cells were calculated from ATIII secretion profiles as shown in Fig. 3B by linear regression and expressed in µg ATIII/$10^6$ cells/d. For all media ATIII concentrations determined by ELISA were corrected for the ATIII concentration present in fresh medium to account for cross-reactivity of the ELISA (Schröder et al., 1996) with bovine ATIII and other serum proteins. Cell line CHO-A11-A279-C7 could not be grown under protein-free conditions (see text for details). Abbreviations: n.a. – not applicable, n.d. – not detectable.

| cell line | specific ATIII secretion rate [µg ATIII/$10^6$ cells/d] in | | |
|---|---|---|---|
| | CHO-T1-SF + 10% (v/v) FCS | CHO-T1-SF + 1% (v/v) FCS | CHO-T1-PF |
| CHO-A11-A2 | n.d. | 0.9 ± 0.2 | 1.23 ± 0.07 |
| CHO-A11-A27 | 4.7 ± 2.3 | 5.2 ± 0.8 | 4.9 ± 0.3 |
| CHO-A11-A279 | 9.2 ± 2.8 | 7.6 ± 1.3 | 7.2 ± 0.3 |
| CHO-A11-A279-C7 | 20.0 ± 4.9 | 29.7 ± 4.3 | n.a. |

**Table 5. Generation times in protein-free medium CHO-T1-PF.** The generation time in the exponential growth phase was calculated by linear regression from the slope of growth curves as shown in Fig. 3. Only the first four to six data points were used for regression analysis.

| Cell line | Generation time [h] |
|---|---|
| CHO DUKXB11 | 20.5 ± 1.5 |
| CHO-A11-A2 | 18.1 ± 0.8 |
| CHO-A11-A27 | 15.7 ± 2.2 |
| CHO-A11-A279 | 37.2 ± 9.2 |