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Propagation of Mammalian Cells and of Virus in a Self-Regulating Fermentor (40852)

BERND FÖHRING,*,[1] SIAN T. TJIA,† W. MARTIN ZENKE,* GERHARD SAUER,* AND WALTER DOERFLER†

Institut für Virusforschung, German Cancer Research Center, 6900 Heidelberg, and †Institute of Genetics, University of Cologne, 5000 Cologne, Germany

Many problems in cell biology and virology make it desirable to propagate tissue culture cells in large quantities. Mass cultivation of mammalian cells has been routinely performed in monolayer and suspension (spinner) cultures. Basically the same culture conditions have been employed to replicate animal viruses in cell culture. The design of most culture vessels in use has prohibited maintenance of virus-infected or uninfected cells under strictly controlled conditions. In studying the specific requirements for optimal cell growth and/or virus replication, a better defined propagation system will be required. A problem of considerable practical importance has been the maintenance of constant pH values particularly in long-term cell culture and in virus-infected cell cultures. Upon virus infection and active virus replication, the pH in cell culture media frequently drops to low values.

There are a number of reports describing how conditions for cell culture (1, 2) and virus production (3, 4) can be optimized. Large scale propagation of mammalian cells has been achieved in several laboratories (5–7).

The present communication outlines technical aspects of the long-term propagation under strictly controlled conditions of a number of permanent cell lines of human or simian origin and of hamster cells transformed by simian virus 40 (SV40). Moreover, the optimal conditions for adenovirus type 2 (Ad2) replication in human KB cells have been determined. Lastly, we show that carrier cultures of papova virus producing Vero cells can be maintained for long periods of time using Cytodex, a beaded microcarrier.

Materials and methods. Cells and viruses. The following cell lines have been used: HeLa, CV-1, Vero, SV40-transformed Chinese hamster cells (CHESV), KB, and HD-virus producing Vero 76 clone A cells (8). Adenovirus type 2 was grown on KB-cells (9). Cells were grown in Joklik's modified minimum essential medium (MEM) supplied by Flow Laboratories and supplemented with 10% fetal bovine serum with the exception of Vero 76 clone A cells which were grown in Eagle's medium supplemented with 5% calf serum.

Fermentor. The self-regulating Braun Biostat S equipped with a 5-liter vessel was used (B. Braun, Melsungen, Germany). Cells were grown at 37°C and a stirring speed of 50 rpm.

Some of the technical aspects of the experiments carried out will be outlined under Results.

Results and discussion. Quantitation of cell density. In order to determine the optimal growth conditions for suspension cultures it is necessary to establish growth curves. Such curves permit an assessment of various parameters that influence the growth of cells. However, the precise determination of cell density in suspension cultures is, depending on the cell type, sometimes hampered by formation of aggregates. Therefore, we considered it important to compare first the suitability of a number of methods with respect to their reproducibility and the ease with which they can be applied.

The determination of cell density with the help of a Coulter counter proved to pose problems owing to aggregate formation. Such aggregates were either registered as individual cells, or, they were ignored if they exceeded a certain size. In general this automated procedure gave an under estimation of the actual cell density. This is

[1] Present address: Department of Pathology, Rutgers Medical School, Piscataway, N.J. 08854.

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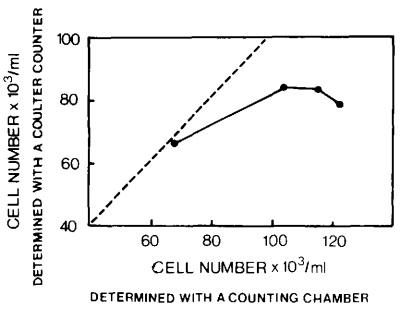

FIG. 1. Determination of cell density with a counting chamber and with a Coulter counter. At various periods of time, samples were removed from a HeLa-suspension culture and the cell density was evaluated by two different methods (●). The dashed line indicates the theoretical values which would be expected if both methods yielded identical results.

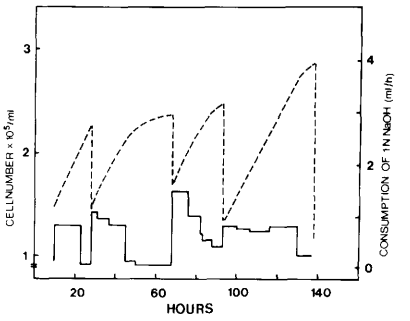

FIG. 3. Batch culture of HeLa cells. The pH in the culture medium was kept constant at pH 7.05 by addition of 1 *N* NaOH using the self-regulating devices of the Biostat S (continuous line). The cell density is indicated by the dashed line. At various periods of time, 1-liter batches comprising half the culture were withdrawn and the withdrawn volume was replaced by fresh medium. Cell numbers were determined in a Neubauer counting chamber.

documented by a comparison of the results obtained by the use of a conventional counting chamber (Neubauer) with those obtained with a Coulter counter (Fig. 1). There is a considerable deviation in the range of higher cell densities which is caused by the inability of the Coulter counter to precisely register aggregates of cells.

Attempts were made to employ the turbidity of samples in which the cells had been homogenized by sonication as a measure for cell density (Fig. 2). The optical density of the culture medium containing sonicated cells was determined at various wavelengths (dashed line). The insert shows the relationship between optical density determined at three different wavelengths and the actual cell density. There is a linear relationship between optical density and cell number at 675 nm which renders this curve particularly useful for reference purposes. During the procedures described in the subsequent sections either turbidity measurements or the counting

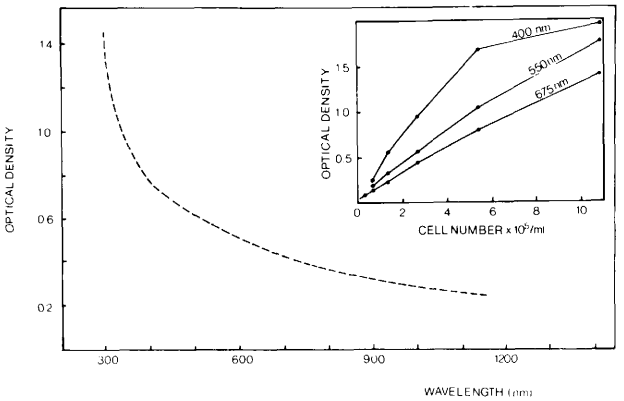

FIG. 2. Determination of cell density by turbidity measurements. CV-1 cells were suspended in MEM, the cell density was determined in a counting chamber, and the cells were then homogenized with a Braun ultrasonic sonifier (30 sec at full power). The optical densities were determined at different wavelengths with a Zeiss PMQ2 photometer using MEM as a reference (inset). The dashed line designates the optical density spectrum of the extracts.

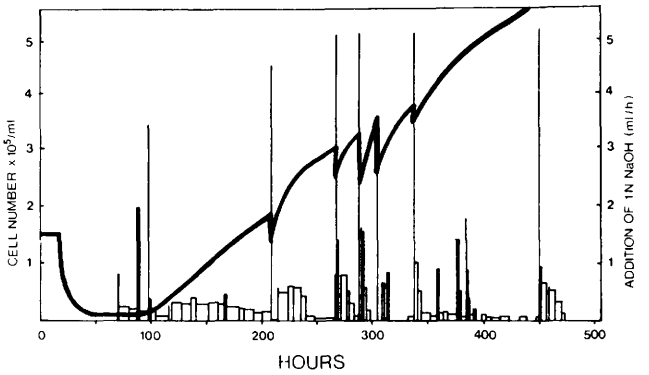

FIG. 4. Batch culture of SV40-transformed Chinese hamster cells. For details see legend to Fig. 3. Bold line: cell density; normal line: amount of NaOH added during titration. The pH of the 2-liter culture was kept constant at pH 7.0. At various periods of time, volumes comprising one-fourth to one-third of the culture were harvested. The volume withdrawn was replaced by fresh medium of the same pH. As CHESV cells tended to form aggregates in the fermentor, cell density was measured by turbidity measurements.

chamber were used as indicated in the figure legends.

The maintenance of optimal growth conditions for cell cultures. All experiments carried out with the Biostat S Fermentor were of the batch type; we have not acquired any experience with continuous cell cultures. It was the aim of this study to establish conditions for optimal cell growth and virus yields. Constancy of the pH value in culture medium turned out to be the most important parameter in determining growth rates of cells and virus yields. The pH value in culture media can be automatically controlled by the addition of alkali (NaOH) to the medium. The graphs presented in Figs. 3 and 4 demonstrate the growth of HeLa cells (Fig. 3) and of SV40-transformed Chinese hamster cells (Fig. 4) at constant pH. Fresh media added to these cultures were kept at the same pH. The amount of 1 *N* NaOH added was recorded and was used as an indirect measurement of cell replication, since the number of cells produced is related within narrow bounds to the amount of alkali required to maintain constant pH

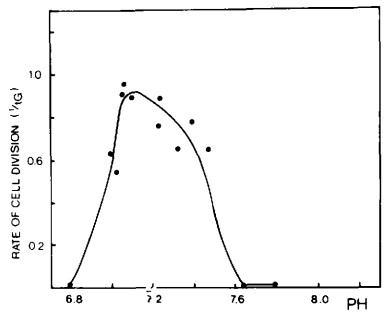

FIG. 5. The influence of pH on the growth of HeLa cells in suspension. Parallel cultures of HeLa cells were maintained at an average density of 2.5 to 4 × 10^5 cells/ml at various pH values in Joklik's modified MEM with 10% fetal bovine serum. The medium was buffered with 30 m*M* Hepes. During a time interval of 18 hr, at the beginning and end of which the cell density was determined, the change of pH did not exceed 0.1 pH unit.

TABLE I. INFLUENCE OF SALT CONCENTRATION ON GROWTH RATE OF HeLa CELLS[a]

NaCl concentration (%)	1/Doubling time (days)
117.5	0.86
115	0.67
110	1.02
100	0.99
90	1.02
85	1.12

[a] HeLa cells were grown in suspension culture in Joklik's modification of Eagle's medium supplemented with 10% fetal calf serum and buffered with 30 m*M* Hepes. Biostat-S fermentor was used, the pH value was controlled at 7.2. The salt concentration was adjusted with NaCl to the values listed; 100% salt concentration corresponds to the NaCl concentration of Joklik's medium.

TABLE II. REPLICATION OF CELL LINES IN SUSPENSION CULTURE[a]

Cell line	Time of doubling (hr)	Optimal cell number[b] (cells/ml × 10^5)
HeLa	24	1–5
KB	46	1–3
CHESV	24	1–5

[a] Cells were grown in the Joklik modification of Eagle's medium supplemented with 10% fetal bovine serum, CHESV cells were grown in Eagle's medium.

[b] These cell densities were reached at the doubling times indicated.

values (cf. Table III). The optimal pH value for the replication of HeLa cells has been determined to be 7.05 (Fig. 5). CHESV cells were maintained at pH 7.0.

Addition of alkali during titration of acid components in the medium will obviously lead to an increase in the ionic strength of the medium. We have tested the effect of NaCl concentration on the growth rate of HeLa cells and found that an increase in salt concentration up to 10% above that in conventional cell culture media did not affect cell replication (Table I). An increase in salt concentration of this magnitude is (by a factor of 0.5) above that expected to arise from titrations as used in the batch procedure described above. Thus, titration of cell culture medium with alkali appears to be a useful procedure in the batch propagation of cells in culture.

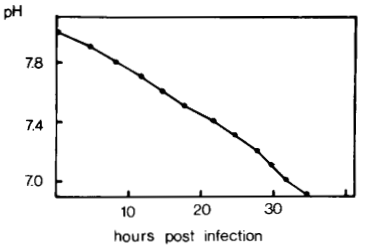

FIG. 6. Change of pH value in an Ad2-infected culture of KB cells. KB cells growing in a 2-liter suspension culture at an initial cell density of 2 × 10^5 cells/ml were infected with a multiplicity of 20 PFU/cell. The actual pH value was registered over a period of 30 to 35 hr postinfection. The pH value was not experimentally controlled.

Growth of cells and virus. Several different continuous cell lines, human HeLa and KB cells, and CHESV, an SV40-transformed Chinese hamster line, have been propagated under controlled conditions in the fermentor by the batch procedure. Doubling times and the optimal cell densities attained for each of the lines have been listed in Table II. A quantitative correlation between the number of CHESV cells and HeLa cells grown in a defined volume of medium and the amount of 1 *N* NaOH required to maintain constancy of pH value has been established. The mean values were 1.1 × 10^7 CHESV cells and 8.5

TABLE III. CORRELATION BETWEEN CELL NUMBER AND AMOUNT OF 1 *N* NaOH NEEDED FOR TITRATION[a]

Experiment No.	Amount of medium (liter)	Mean cell density per ml (×10^5)	Total cell number per ml 1 *N* NaOH added (×10^6)
CHESV cells			
1	0.5	1	9.8
2	0.5	2–3	10.8
3	0.5	2–3	13.6
HeLa cells			
1	1	1.5	10.8
2	0.5	2–3	6.7
3	0.5	2–3	7.1
4	0.5	2–3	9.5

[a] CHESV cells were grown in Eagle's medium, HeLa cells in Joklik's modification of Eagle's medium. All values were measured during logarithmic growth. Density of CHESV cells was determined by turbidity measurements, that of HeLa cells in a Neubauer counting chamber.

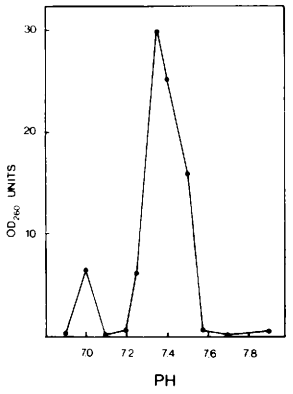

FIG. 7. Ad2 yields from KB suspension cultures maintained at strictly controlled pH values in a Biostat S fermentor. Ad2-infected cultures (2 liters) were harvested at 37 hr postinfection and virus from 0.5-liter portions was harvested and purified as described elsewhere (9). Upon two cycles of equilibrium centrifugation in CsCl density gradients, the total amount of virus (ρ = 1.334 g × cm^{-3}) was determined in OD_{260} units (1 OD_{260} unit = 1 ml of virus suspension at OD_{260} = 1.0. One OD_{260} unit corresponds to about 10^{10} PFU). In each experiment 2-liter cultures were maintained for 35 hr at the pH values indicated.

× 10^6 HeLa cells per milliliter of NaOH added for titration (Table III).

Human adenovirus type 2 (Ad2) is routinely propagated to high titers in suspension cultures of human KB cells. It has been known for a long time, that in unregulated cultures of Ad2-infected KB cells the pH value drops rather markedly. In the experiment documented in Fig.6, within a period of 30 to 35 hr the pH dropped from a value of 8 to 6.9. It is apparent from the data summarized in Fig. 7 that the yields of Ad2 obtained in suspension culture are highly dependent upon the pH in the culture medium. For Ad2 a rather sharp optimum was found at pH 7.35 (Fig. 7). Such dependence of the yield of various viruses on the pH in the medium was previously reported by Fields and Eagle (3). We have also measured the amounts of incomplete particles of adenovirus type 2 (10) produced in cultures maintained at pH values between 7.3 and 7.9, but have not found a clear dependence on pH. The amounts of viral components produced were not determined.

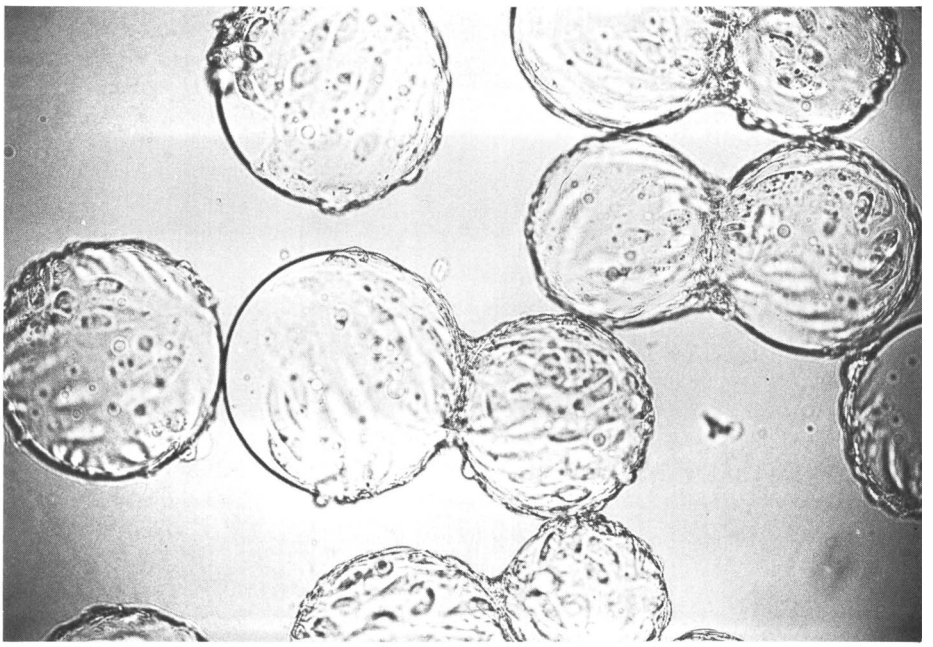

FIG. 8. Photomicrograph of Vero 76 clone A cells latently infected with HD virus on Cytodex 1 beaded microcarrier at 11 days after seeding (see Fig. 9).

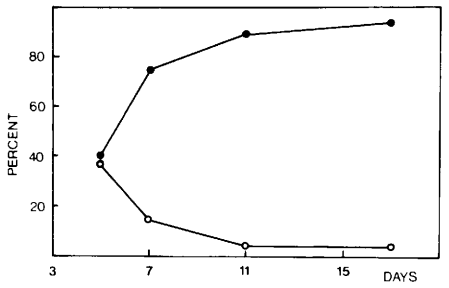

FIG. 9. Growth of Vero 76 cells on Cytodex 1 beads. Vero 76 clone A cells (a clonal line of HD-virus producing Vero 76 cells) were seeded together with 5 g of Cytodex 1 (Pharmacia) in 2 liters of Eagle's suspension culture medium containing 5% calf serum (4×10^4 cells/ml). Cells were cultivated in the Braun Biostat S fermentor. After 5 days, the culture volume was increased to 3 liters by addition of medium. At time intervals indicated, samples were withdrawn and between 100 to 200 beads were examined for the presence of cells. Beads with less than five cells were scored as negative (○), while the beads with more than five cells were scored as positive (●).

Growth of persistently virus infected cells on microcarrier beads. The automated cell culture fermentor was applied to grow virus-producing cells on the beaded microcarrier Cytodex 1 (Pharmacia, Uppsala, Sweden). The virus-synthesizing Vero 76 cell system (8, 11), which we employed, is particularly suitable for this purpose, as the virus–host cell interaction in the persistently infected Vero 76 cell line is not cytocidal. Rather, the production of viral components such as capsid proteins and viral superhelical DNA progresses at a slow rate, allowing growth of the cells on the microcarrier beads (Fig. 8). The data in Fig. 9 show that microcarrier beads are being covered with cells during the period of incubation in the fermentor. At the same time, the proportion of cell-free beads is reduced. On the 11th day after establishing the suspension culture, a sample of Vero 76 cells was harvested. By comparative agarose-gel electrophoresis we could demonstrate the presence of HD-viral superhelical DNA in these cells (W. M. Zenke and G. Sauer, unpublished data). Thus, persistently infected papovavirus-producing cells can be cultured on microcarrier beads in the fermentor. We have not yet determined whether the yield of viral DNA exceeds the values obtained from conventional monolayer Vero 76 cultures. The experimental conditions described allow the maintenance of virus-producing cultures in the automated fermentor for prolonged periods of time. When required, batches of virus-producing cells can be harvested for preparation of viral components, while the remainder of the culture is being further cultivated after replacement of the withdrawn volume by fresh culture medium.

Conclusions. We have shown that defined conditions of cell maintenance, in particular the constancy of pH values, are of great importance for optimal cell growth and virus yields. The automated fermentor ensures controlled conditions in large volume cultures. This equipment should prove useful in large scale cell and virus propagation, particular in combination with microcarrier systems.

Summary. A number of different mammalian cell lines were cultivated in large scale batches in a fermentor which controlled the pH value of the medium. Acid produced due to cellular metabolism was titrated by the addition of NaOH. It was shown that the ensuing increase in NaCl concentration had no deleterious effects on cell growth, as long as this increase did not exceed 10% of the regular NaCl concentration in the medium.

The production of human adenovirus type 2 (Ad2) in large scale suspension cultures of KB cells was found to be strongly pH dependent; optimal yields were obtained at pH 7.35. Under uncontrolled conditions the pH value in an Ad2-infected culture of KB cells dropped from 8 to 6.9 within a period of 35 hr.

Monkey Vero cells latently infected with the papova HD virus strain of stump-tailed macaque virus were propagated on a beaded microcarrier in the fermentor. It was shown that such microcarrier complexes were capable of producing viral DNA.

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