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Improvements in the Growth of BHK-21 Cells in Submerged Culture

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A medium is described which will support the submerged culture of BHK-21 cells to 6.5 × 10^6 to 7 × 10^6 cells/ml in volumes up to 30 liters. Shaken-flask cultures were used to determine nutrient deficiencies in depleted medium. The final medium became limited by glutamine at 6.5 × 10^6 to 7 × 10^6 cells/ml, but increasing the glutamine concentration failed to improve cell yields. The value of the polyol Pluronic F68 as a protective substance is illustrated.

The usefulness of the baby hamster kidney cell (BHK-21) in the commercial production of foot-and-mouth disease vaccine has encouraged further work on increasing cell yields in submerged culture. In early work at Pirbright, yields of 2 × 10^6 to 3 × 10^6 cells/ml were obtained (8, 9, 11). More recently, D'Oultremont et al. (2) and Zoletto and Gagliardi (13) described yields of 4 × 10^6 to 6 × 10^6 cells/ml with media containing lactalbumin hydrolysate, but their published data suggest that exponential growth ceased at 2 × 10^6 and 4 × 10^6 cells/ml, respectively. The highest yields were reported by Zoletto and Gagliardi, who obtained a 12-fold increase of cells to a maximum of 6 × 10^6 cells/ml in a culture period of 96 hr.

In our laboratory, a medium (ZG) made to the formulation of Zoletto and Gagliardi gave improved but variable results at the 4-liter scale, with an average maximum density of 3.5 × 10^6 cells/ml (coefficient of variation, 20%), but, at the 30-liter scale, yields were consistently low, at only 2 × 10^6 cells/ml. These differences prompted further study. This report outlines the results of our investigations and the subsequent development of a medium giving cell densities of 6.5 × 10^6 to 7 × 10^6 cells/ml in culture volumes up to 30 liters.

MATERIALS AND METHODS

Culture vessels and conditions of culture. The culture vessels used were stirred vessels with working volumes of 4 and 30 liters fitted with automatic systems for the control of temperature and *p*H value. The general design of this equipment was described previously (9, 11). Starting cell concentrations were approximately 0.5 × 10^6 cells/ml, and incubation temperature was 35 C. In the preliminary stages of this work, *p*H 7.4 was maintained by the automatic injection of air and CO_2 gas as described by Telling and Stone (11). A difficulty of this system was its failure to control *p*H when cell growth reached about 3 × 10^6 cells/ml. When *p*H control was restored by the addition of a further 2.2 g of sodium bicarbonate per liter, dissolved oxygen tension fell rapidly to zero, and there was little further cell growth. To overcome this problem, we adopted an empirical method. The control point of *p*H 7.4 was set in the usual manner, but, on loss of control, it was allowed to fall to *p*H 7.2 before the addition of 1.0 g of sodium bicarbonate per liter. This procedure was satisfactory but made frequent inspection of the culture essential, and the method was eventually replaced by acid/alkali control with 1.0 M HCl and 1.0 M Na_2CO_3. Cultures controlled in this manner were aerated by continuously sparging a low flow rate of air, which was adjusted at intervals. The flow rates used were calculated on the basis of the cell density and the oxygen solution rates obtainable in the culture vessel from different flow rates of air. This method has been described briefly by Telling and Radlett (10) and in detail by Radlett et al. (*in preparation*).

Shaken-flask cultures. These were used to determine both nutritional deficiencies in depleted medium from the stirred culture vessels and the maximum nontoxic concentration of glutamine. Culture volumes were 200 ml in 500-ml capacity Erlenmeyer flasks which were seeded at 0.5 × 10^6 cells/ml, incubated at 37 C, and agitated at 200 oscillations/min on an orbital shaker (model G10-20-500, New Brunswick Scientific Co.). When the culture reaction fell below *p*H 7.1, correction to *p*H 7.2 to 7.4 was made by adding sterile sodium bicarbonate. For work on depleted medium, cultures which had reached peak cell densities were freed of cells by filtration. The cell-free filtrates were supplemented with fresh medium components both singly and in combinations and tested for their ability to support further cell growth.

Assays. Cell counts were made with a Fuchs-

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TABLE 1. *Development of improved medium at the 30-liter scale*

Type of medium	Concn of some important constituents						Peak viable cells[c]	Yield (10^6 cells produced per mg of glucose used)
	Edifas B (g/liter)	P1-F68[a] (g/liter)	Glutamine (g/liter)	Glucose (g/liter)	Vitamin concentrate (ml/liter)[b]	Lactalbumin hydrolysate (g/liter)		
ZG	1.0		0.23	3.6	3.2	2.5	2.0	0.90
		1.0	0.23	3.6	3.2	2.5	2.7	0.90
		1.0	0.47	3.6	3.2	2.5	3.5	0.90
		1.0	0.47	6.2	3.2	2.5	4.4	0.95
6M		1.0	0.47	6.2	6.4	2.5	5.5	1.10
7M		1.0	0.93	9.2[d]	6.4	5.0	7.0	1.00
		1.0	1.40	9.2[d]	8.0	5.0	6.7	0.90

[a] Pluronic polyol F68.
[b] Final concentration of each vitamin is given in Table 3.
[c] Values expressed $\times 10^{-6}$/ml.
[d] At start, 6.2 g of glucose per liter present; 3.0 g/liter added at about 45 hr.

TABLE 2. *Growth of BHK cells in shaken-flask cultures containing depleted medium*

Nature of supplement	Peak viable cells[a]	
	In depleted 6M	In depleted 7M
Glutamine	0.6	1.5
Vitamins	0.5	0.9
Lactalbumin hydrolysate	0.8	0.8
Glutamine and vitamins	0.8	1.8
Glutamine and lactalbumin hydrolysate	1.1	1.4
Vitamins and lactalbumin hydrolysate	0.7	0.9
Glutamine, vitamins, and lactalbumin hydrolysate	1.2	1.2

[a] Values expressed $\times 10^{-6}$/ml.

Rosenthal counting chamber, and viability was determined by trypan blue permeability. An enzymatic technique based on the methods of Marks (5) and Washko and Rice (12) was used for glucose estimation.

RESULTS

Supplementation studies. In our hands, depleted ZG medium was found to be growth-limited for glutamine. Increasing the concentration of glutamine in the culture medium resulted in improved cell densities and exhaustion of glucose. Peak cell densities of more than 4×10^6 cells/ml were obtained by increasing the starting glucose concentrations to 6.2 g/liter, and, when the concentration of vitamins was doubled, average peak cell densities of 5.5×10^6 cells/ml were achieved. This medium was designated 6M, and these results are shown in Table 1. Shaken-flask cultures were prepared with depleted 6M medium, and the results presented in Table 2 indicate that lactalbumin hydrolysate and glutamine were the growth-limiting factors. It was also apparent from the cell yields from glucose that, to achieve cell concentrations above 6×10^6 cells/ml, additional glucose was required. We found, however, that when the starting glucose concentration exceeded 6.2 g/liter, cell yields were reduced both in culture vessels and in shaken flasks, even though the tonicity was adjusted. Hence, it became necessary to add glucose during the culture period. These modifications resulted in a formulation designated 7M, which yields 6.5 to 7×10^6 cells/ml in about 63 hr, with a viability of 95%. Shaken-flask cultures with depleted 7M medium indicated that glutamine and possibly vitamins were growth-limiting factors (Table 2), but extra glutamine and vitamins added to 7M medium did not result in improved cell densities (Table 1). In 7M medium, growth becomes exponential after 10 to 12 hr, with an average growth rate constant of 0.05 hr^{-1}, showing that under cultural conditions which are not rate-limiting the BHK cell has an approximate doubling time of 14 hr. The formulation of 7M medium is shown in Table 3, and a typical growth curve is given in Fig. 1.

Pluronic polyol F68. Several workers have reported cell damage as a result of excessive agitation or sparging. Pluronic polyol F68 (P1-F68) is a nonionic surface-active polymer which has been reported to protect mammalian cells against this type of damage, probably by stabilizing the cell-liquid interface (4, 7). Early in this work, the influence of concentration of P1-F68 on BHK cell growth was examined at the 4-liter scale, and results (Table 4) indicated that peak cell densities were maximal when it was incorporated into medium at a concentration of 1 g/liter. The

TABLE 3. *Pirbright 7M medium for submerged culture of BHK-21 cells*

Component	Amt (mg/liter)	Component	Amt (mg/liter)
NaCl	5,120	L-Arginine	8.4
KCl	321	L-Cystine	4.8
$MgSO_4 \cdot 7H_2O$	160	L-Histidine	3.84
$NaH_2PO_4 \cdot 7H_2O$	112	L-Isoleucine	10.48
$CaCl_2$	160	L-Leucine	10.48
$Fe(NO_3)_3 \cdot 9H_2O$	0.08	L-Lysine HCl	14.62
$NaHCO_3$	2,200	L-Phenylalanine	6.6
Glucose	6,200[a]	L-Threonine	9.52
TPB Powder[b]	2,000	L-Tryptophan	1.6
Phenol red	8	L-Tyrosine	7.24
Riboflavine	0.32	L-Valine	9.36
Choline chloride[c]	3.2	L-Methionine	3.0
Folic acid	3.2	L-Glutamine	932
Nictonamide	3.2	Inositol	0.7
Pyridoxal HCl	3.2	Pluronic F68[d]	1,000
Thiamine (aneurine) HCl	3.2	Lactalbumin hydrolysate[e]	5,000
DL-Pantothenic acid calcium salt	3.2	Bovine serum	100[f]
		Antifoam emulsion[c]	0.4[f]

[a] Additional 3,000 mg/liter required at about 45 hr.
[b] Difco, Detroit, Mich.
[c] Hopkin & Williams Ltd., Chadwell Heath Essex, U.K. All other vitamins and amino acids from Koch-Light Laboratories, Ltd., Colnbrook, Bucks, U.K.
[d] Wyandotte Chemicals Corp., Wyandotte, Mich.
[e] Nutritional Biochemical Corp., Cleveland, Ohio.
[f] Expressed as milliliters.

improvement in cell growth obtained by incorporating 1 g of P1-F68 per liter into ZG medium in place of Edifas B at the 30-liter scale is shown in Table 1.

Toxicity of glutamine. Griffiths and Pirt (3) reported that glutamine was toxic to their mouse LS cells at concentrations exceeding 10 mM. Since glutamine was the growth-limiting nutrient at several stages of this work, it was clearly desirable to examine the toxicity of glutamine to BHK cells. Table 5 shows the result of shaken-flask cultures in which cells were grown in modified Eagle's medium (9) containing various concentrations of glutamine. The reduction in peak cell yields when glutamine concentration exceeded 10 mM (1.46 g/liter) suggests that the observation of Griffiths and Pirt is also applicable to BHK cells.

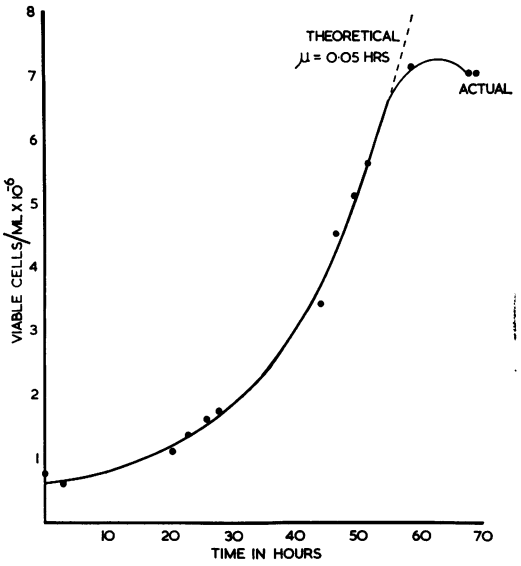

FIG. 1. *Growth curve of BHK cells in 7M medium.*

TABLE 4. *Effect of concentration of Pluronic polyol F68 (P1-F68) on cell growth at the 4-liter scale*

Concn of P1F68 (g/liter)	Peak viable cells[a]	Viability at peak cells (%)	Yield (10^6 cells produced per mg of glucose used)
0	1.85	86	0.45
0.125	2.45	82	0.50
0.5	3.23	91	0.60
0.75	3.45	92	0.70
1.0	4.84	97	1.20
1.5	3.62	97	0.80
2.0	3.25	94	0.60

[a] Values expressed $\times 10^{-6}$ per ml.

TABLE 5. *Effect of glutamine concentration on cell growth in shaken flasks*

Glutamine concn (mmoles/liter)	Peak viable cells ($\times 10^{-6}$/ml)
0	0.50
1.6	0.94
5.0	0.99
10.0	1.31
12.0	1.19
15.0	0.92
20.0	0.49

DISCUSSION

The use of lactalbumin hydrolysate as a medium component, combined with increased concentrations of other medium constituents, enabled us to increase cell densities from 2×10^6 to 3×10^6 cells/ml to 6.5×10^6 to 7×10^6 cells/ml

in volumes up to 30 liters. Cell growth was exponential after 12 hr in culture until about 55 hr, when growth rate became limited, apparently by the glutamine concentration. According to the analysis provided by the manufacturers, we could expect lactalbumin hydrolysate to contain 12 to 18% glutamine. Medium containing 5 g of lactalbumin hydrolysate per liter could therefore contain 4 to 6 mmoles of glutamine per liter from this source alone, and the total glutamine concentration could be as high as 10 to 12 mM. This could explain why supplementation of 7M failed to improve cell densities further and suggests that greater densities might be obtained by dosing the culture with glutamine at intervals.

Griffiths and Pirt (3) found, for their mouse LS cells, that glutamic acid could be substituted for glutamine after a suitable induction period, but our attempts to grow BHK cells on glutamate failed even after several medium changes. De Mars (1) and Paul and Fottrell (6) both reported repression of glutamine synthetase by glutamine, and our failure to induce cell growth on glutamate may have resulted from the presence of glutamine in lactalbumin hydrolysate.

We are unable to explain our need to supplement ZG medium to achieve maximum cell densities in excess of 3.5×10^6/ml. Possible reasons are variations in environmental conditions and the use of constituents from different sources. Whatever the cause, this report illustrates the difficulty of obtaining reproducible results between laboratories.

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LITERATURE CITED

1. De Mars, R. 1958. The inhibition by glutamine of glutamyl transferase formation in cultures of human cells. Biochim. Biophys. Acta 27:435–436.
2. D'Oultremont, Ph. A., B. Guilloteau, A. Colleau, B. Legrand, and P. Prunet. 1968. Adaptation de la cellule BHK 21 à la culture en suspension. Paper presented at the 12th Conference of the F.M.D. Commission of O.I.E., Paris.
3. Griffiths, J. B., and S. J. Pirt. 1967. The uptake of amino acids by mouse cells (strain LS) during growth in batch culture and chemostat culture: the influence of cell growth rate. Proc. Roy. Soc. Ser. B. Biol. Sci. 168:421–438.
4. Kilburn, D. G., and F. C. Webb. 1968. The cultivation of animal cells at controlled dissolved oxygen partial pressure. Biotechnol. Bioeng. 10:801–814.
5. Marks, V. 1959. An improved glucose-oxidase method for determining blood, C.S.F. and urine glucose levels. Clin. Chim. Acta 4:395–400.
6. Paul, J., and P. F. Fottrell. 1963. Mechanism of D-glutamyl transferase repression in mammalian cells. Biochim. Biophys. Acta 67:334–336.
7. Runyon, W. S., and R. P. Geyer. 1963. Growth of L cell suspensions in a Warburg apparatus. Proc. Soc. Exp. Biol. Med. 112:1027–1030.
8. Telling, R. C. 1969. Foot and mouth disease vaccine. Process Biochem. 4 (no. 6):49–52.
9. Telling, R. C., and R. Elsworth. 1965. Submerged culture of hamster kidney cells in a stainless steel vessel. Biotechnol. Bioeng. 7:417–434.
10. Telling, R. C., and P. J. Radlett. 1970. Large scale cultivation of mammalian cells. Adv. Appl. Microbiol. 13:91–119.
11. Telling, R. C., and C. J. Stone. 1964. A method of automatic *p*H control of a bicarbonate-CO_2 buffer system for the submerged culture of hamster kidney cells. Biotechnol. Bioeng. 6:147–158.
12. Washko, M. E., and E. W. Rice. 1961. Determination of glucose by an improved enzymatic procedure. Clin. Chem. 7:542–545.
13. Zoletto, R., and G. Gagliardi. 1968. Lactalbumin hydrolysate for the growth of BHK 21 Cl13 cells in suspension. Paper presented at the 16th Venetian Regional Conference of the Italian Association of Hygiene and Public Health.