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Production and Purification of Milligram Amounts of Foot-and-Mouth Disease Virus From Baby Hamster Kidney Cell Cultures

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ABSTRACT

POLATNICK, J. (Plum Island Animal Disease Laboratory, Greenport, N.Y.), and HOWARD L. BACHRACH. Production and purification of milligram amounts of foot-and-mouth disease virus from baby hamster kidney cell cultures. Appl. Microbiol. **12**:368-373. 1964.—A stable line of baby hamster kidney cells for use in the production of, and subsequent purification of, foot-and-mouth disease virus (FMDV) was grown in large quantities on the cylindrical surfaces of 2-liter Baxter bottles. The bottles, in round wire cages, were rotated on a three-tiered roller mill. The cells retained their rapid growth characteristics and susceptibility to FMDV in a tris(hydroxymethyl)aminomethane buffer-containing medium which was especially formulated for large-scale work. This medium, without being changed, sustained cell growth for 6 to 7 days to yield confluent layers containing 500 to 750 million cells per bottle. In small-scale virus-growth experiments, harvested fluids contained about $10^{3.8}$ to $10^{8.8}$ plaque-forming units (PFU) per ml. This corresponded to a yield of 30 to 50 PFU per cell. In production runs with 190 cultures, the infectious fluids usually contained $10^{7.9}$ to $10^{9.2}$ PFU per ml, and the mass of essentially pure virus obtained therefrom ranged from 7 to 17 mg concomitant with cumulative infectivity recoveries of about 20%.

Primary bovine kidney cultures have been used in this laboratory for several years to produce and quantitate foot-and-mouth disease virus (FMDV). Such cultures have been very useful but, nevertheless, have the known disadvantages of slow growth, a heterogeneous cell population, and nonuniform susceptibility to infection. In addition, the cultures for virus production are cumbersome to handle and uneconomical to prepare in large numbers, and sterility is uncertain.

A rapidly growing stable cell line of nontumor origin which grows to high concentrations and which yields high titers of virus upon infection would be useful for large-scale production of virus. A promising cell line from baby hamster bidney (BHK) has been reported (MacPherson and Stoker, 1962; Mowat and Chapman, 1962; Rivenson and Segura, 1963). However, the medium used, because of its requirement for CO_2, low buffering capacity, and costly purified amino acids, is not adaptable to mass culturing and subsequent virus infection. The present report describes media, techniques, and apparatus which permit large-scale growth of BHK cells for use in producing milligram quantities of virtually pure FMDV.

MATERIALS AND METHODS

Apparatus and media for growth of cells. Uncloned BHK cells, supplied by M. Stoker, Institute of Virology, University of Glasgow, Glasgow, Scotland, were initially grown in our laboratory in 4-oz prescription bottles by use of media which he described (MacPherson and Stoker, 1962). Later cultures with altered media were grown in cylindrical 2-liter Baxter bottles (Leunen, Mammerickx, and Strobbe, 1963) held in round wire cages and rotated on a three-tiered roller mill (Fig. 1). This mill had been used for growing bovine kidney cultures in this laboratory (Bachrach, 1964), and a similar one had been used for the same purpose by Ubertini et al. (1963). These bottles required no special cleaning. Each of the 12 cages held 19 bottles. To insure an even distribution and attachment of the cells around the glass surface, a rotational speed of about 3 rev/min was maintained for an initial 2-hr period. The speed was then decreased to 2 rev/hr for growth periods of 6 and 7 days prior to infection and repassage, respectively. Media are, in part, the subject of this investigation, and are developed in the experiments. With the cell-media system employed there was no requirement for a fluid change during cell growth.

Passage of cells. Growth medium was removed from 7-day-old cell layers in Baxter bottles and replaced with 20 ml of 0.25% trypsin in phosphate-buffered saline at about pH 7.5. In the presence of trypsin, 20 min of rotation at room temperature were required to detach the cells. They were then suspended in fresh growth medium and dispensed in 100-ml quantities at a concentration of about 300,000 cells per ml to yield 20 new cultures from each old one. Cell concentrations were determined by hemocytometer count, and cell viability was determined by staining with trypan blue (McLimans et al., 1957).

Virus production. FMDV type A strain 119, passaged in bovine kidney tissue cultures 109 times, was stored at −60 C for use as stock virus. Type C strain 3-Canefa, obtained from cattle in Argentina, was adapted directly

to BHK cell culture. BHK cells (6-day-old) in Baxter bottles were incubated with suitably diluted viral inoculum for 30 min at 3 rev/min. Additional fluid was then added, when required, and rotation was slowed to 2 rev/hr. After incubation at 37 C for a specified time, the infectious fluids from all bottles in an entire cage were harvested simultaneously by draining through a large stainless-steel funnel into a chilled carboy (Fig. 2). Lifting of the cages was facilitated by pulleys and inversion of the cages by a rotatable support mounted over the funnel. Plaque-forming units (PFU) of infectivity were assayed in bovine kidney cultures (Bachrach et al., 1957). Virus mass and integrity of the physical particle were determined spectrophotometrically (Bachrach, Trautman, and Breese, 1964; Bachrach, 1964).

RESULTS

Medium for cell growth. The growth medium of MacPherson and Stoker (1962), consisting of 80% Eagle's medium, 10% Tryptose Phosphate (Difco), and 10% calf serum, required continuous control of the gas phase to maintain its bicarbonate buffer capacity. Otherwise, the pH of the media rose to toxic levels. This difficulty was circumvented, as described herein, by buffering the Eagle's salt solution principally with 0.02 M tris(hydroxymethyl)-aminomethane (tris). Sodium chloride was added to maintain isotonicity, and some of the sodium bicarbonate was retained as possible substrate for the cells. The costly purified amino acids in Eagle's medium were replaced by lactalbumin hydrolysate fortified with histidine to compensate for an amino acid deficiency. Table 1 gives the composition of our growth medium (GM). The proportions of modified Eagle's salt solution, tryptose phosphate, and calf serum in the GM were the same as were used by MacPherson and Stoker.

Growth pattern of BHK cells. Table 2 gives the cell concentrations and pH values in BHK cultures up to 7 days after seeding. The pH rose for the first day and dropped thereafter. The pH pattern was unchanged when loose aluminum-foil caps were used, indicating that predominantly nonvolatile acids were produced. Cell viability at 7 days was greater than 95%.

Cellular yield versus inoculum size determinations were made to establish conditions which would give undegenerating maximal cell populations on the seventh day without fluid change during the growth period. For a 30 million to 40 million cell inoculum, the number of cells growing on the glass surface after 2 days surpassed the number initially added. Without medium change, the cells reached an undegenerating population of 500 to 750 million in 6 days—the age at which they were infected. The 10 million cell inoculum likewise yielded actively proliferating cultures, but a longer time was required to reach a given population.

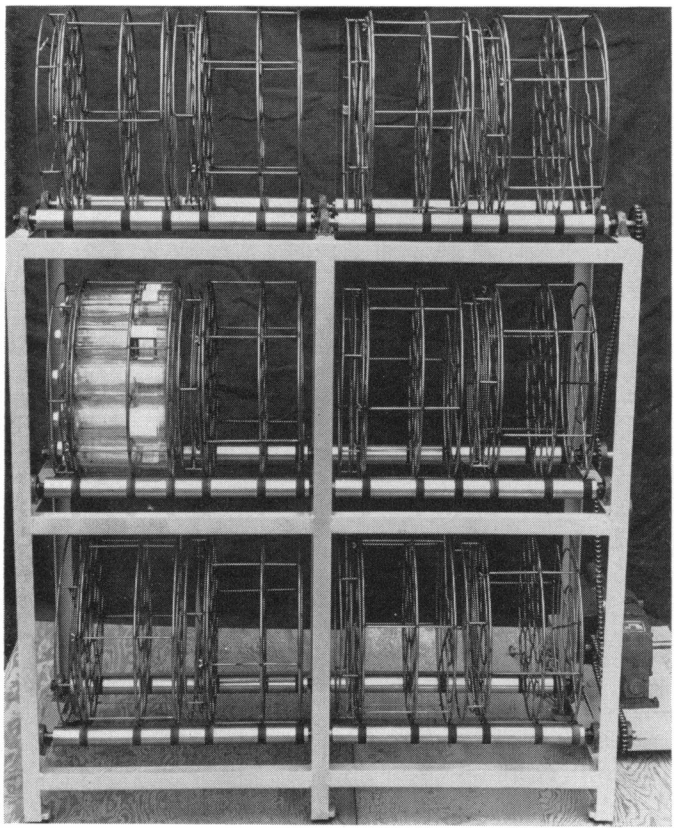

FIG. 1. *Three-tiered roller mill for culturing BHK cells. Capacity: 19 Baxter bottles (2-liter) per cage, 228 cultures total.*

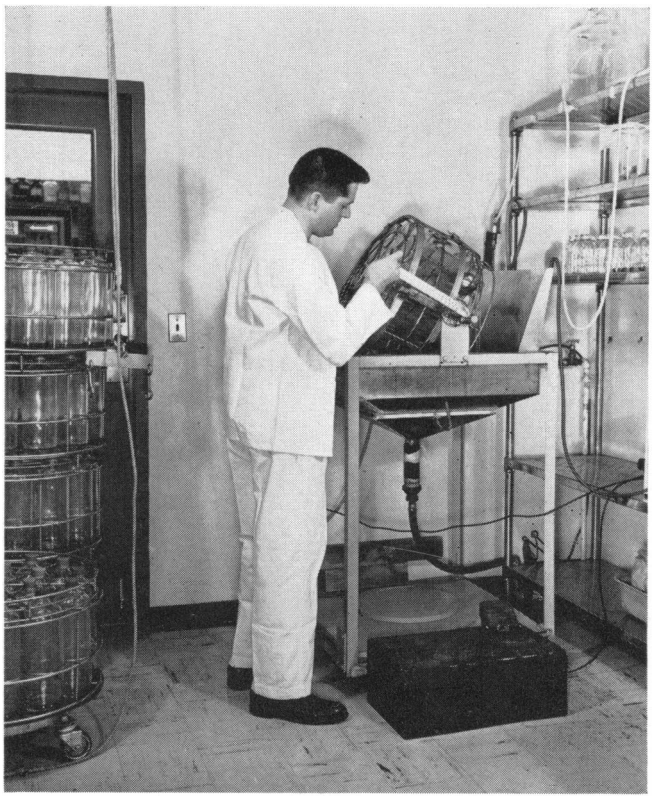

FIG. 2. *Stainless-steel funnel with rotatable mount for pouring off fluids. Rubber tubing shown for carrying spent culture fluid to floor drain is replaced with chilled carboy for harvesting infectious fluids.*

The 160 million cell inoculum shortened the time required for maximal growth to about 4 days, but growth slowed greatly thereafter with cellular degeneration apparent at the seventh day.

Appearance and distribution of virus. The rate of synthesis of FMDV in BHK cells in 4-oz prescription bottles is shown in Table 3. The medium was a 1:1 mixture of GM and 0.16 M tris (GM-tris). Intracellular virus, which first appeared at 160 min postinoculation, was released into the medium 75 min later. Maximal titers were reached at 24 hr, with most of the virus still being intracellular. About 33 PFU were produced per cell.

Inoculum size and incubation fluid. For use in the purification of large quantities of virus, it was desired that virus be released from the BHK cells into the smallest volume possible. It was known from work with bovine kidney cells that 15 ml of media covered the culture layer adequately during rotation on the mill (Bachrach, 1964). Unfortunately, 15 ml of GM or even GM-tris did not hold the pH of the five- to sevenfold greater number of infected BHK cells in the range of FMDV stability. Thus, experiments (Table 4) were carried out to determine the kind and amount of medium required to hold the pH of infected cultures between 7.0 and 7.3 for maximal virus yield. In experiment 1, there was only the single addition of GM-tris medium containing the infecting virus. In the later experiments, the virus originally added in GM was followed after 30 min by the medium shown. It was concluded that original inocula varying from 1 to 15 ml, when fortified after 30 min with sufficient GM-tris medium to make 50 ml, gave the best virus yields. The 50% lower yields from single inocula of 25 or 50 ml in GM-tris would still be useful, however, in large-scale work where double handling is impractical or even impossible.

Contrary to results in prescription bottles, virus in Baxter bottle cultures was almost quantitatively in the super-

TABLE 1. *Growth medium (GM)**

Proportion	Component	Amt per liter
%		
80	Modified Eagle's medium	
	NaCl	6.85 g
	KCl	0.4 g
	$CaCl_2$	0.2 g
	$MgSO_4 \cdot 7H_2O$	0.2 g
	$NaH_2PO_4 \cdot H_2O$	0.12 g
	$Fe(NO_3)_3 \cdot 9H_2O$	0.0001 g
	Glucose	4.5 g
	L-Glutamine	0.292 g
	$NaHCO_3$	0.35 g
	Tris buffer (pH 7.5, 0.16 M)	125.0 ml
	Lactalbumin hydrolysate	5.0 g
	L-Histidine·HCl	0.05 g
	Vitamins (Difco, Hela)	0.2 g
	Phenol red (10%)	0.15 ml
10	Tryptose phosphate broth (Difco)	
10	Bovine serum	

* Containing penicillin, dihydrostreptomycin, and nystatin, each at 100 units per ml.

TABLE 2. *Rate of growth of baby hamster kidney cells and pH changes*

Day	pH*	No. of cells ($\times 10^7$) after initial inoculum of		
		10^7 cells	3.0–4.0 $\times 10^7$ cells	16.0 $\times 10^7$ cells
1	7.65	—	1.6	—
2	7.26	—	6.2	—
3	6.95	5.5	29.3	49.9
4	6.87	20.3	45.2	60.2
5	6.85	37.8	57.7	61.2
6	6.79	49.6	68.8†	64.8
7	6.72	60.0	81.0	44.4

* The initial pH was 7.4 to 7.5. Changes recorded are for the inoculum of 3.0–4.0 $\times 10^7$ cells.

† Range was from 50 to 75 $\times 10^7$ cells compared with 5 to 15 $\times 10^7$ cells in primary bovine kidney cultures.

TABLE 3. *Virus production: distribution between fluid and cells**

Time after inoculation	Log PFU per ml in	
	Fluid	Cells
120 min	—	2.2
150 min	—	2.3
155 min	—	2.2
160 min	—	2.7
165 min	2.2	3.2
220 min	2.2	—
225 min	2.4	—
230 min	2.3	—
235 min	3.1	—
300 min	5.0	6.6
12 hr	6.2	6.9
24 hr	6.8	7.3
30 hr	6.5	7.0

* Multiplicity of infection was 0.1 to 2.0. Unadsorbed viral inoculum was removed by five tris-buffer rinses. Cells were lysed with 0.5% sodium lauryl sulfate in a volume equal to that of the supernatant fluid.

TABLE 4. *Effect of media composition and volume on virus yield*

Expt	Original virus inoculum* (ml)	Medium added after 30 min to make 50 ml total†	Virus yield
			log PFU/ml
1	GM-tris (15)	None	7.9
	GM-tris (25)	None	8.4
	GM-tris (50)	None	8.2
2	GM (5)	GM	8.0
	GM (5)	GM-tris, 3:1	8.2
	GM (5)	GM-tris	8.5
3	GM (1)	GM-tris	8.5
	GM (2)	GM-tris	8.4
	GM (15)	GM-tris	8.5

* Multiplicity of infection was 0.017; harvest time was 24 hr.

† Serum was 10% of the total volume; GM-tris was 1:1; tris was 0.16 M.

natant fluid at 24 hr. This is based on the fact that infected cells lysed with sodium lauryl sulfate yielded little or no additional infectivity. It would appear that mechanical turbulence in the fluid during rotation aids in rupturing infected cells and in releasing virus.

Multiplicity of infection and incubation time. Essentially identical virus yields of $10^{8.4}$ PFU per ml were obtained with multiplicities ranging from 0.001 to 3.0 PFU per cell. Lower multiplicities gave smaller virus yields. Thus, 0.01 to 0.05 PFU per cell were routinely used to allow for uncertainties in cell count and virus titer. The optimal incubation time prior to virus harvest was 20 to 26 hr (Fig. 3).

Culture age and number of cells. Virus yield was determined as a function of both age of the cultures and number of cells. The PFU per cell ratio either increased or remained constant with increasing cell numbers and culture age (Table 5). The reason for low yield on days 3, 4, and 5 in experiment 2 is obscure, but it was, nevertheless, within the normal range by the sixth day. A 6-day period of growth prior to infection was the most convenient for larger-scale operations and, fortunately, gave maximal virus in all experiments.

Simplification of virus production medium. Virus was produced in the foregoing experiments in the presence of 10% calf serum, vitamins, and glutamine. For larger-scale production and for virus purification, lesser amounts of proteins and other organic materials were desired. It was found (Table 6) that the initial 15 ml of inoculum in GM-tris without vitamins or glutamine and only 1 or 5% serum, followed after 30 min by 35 ml of GM-tris without vitamins, glutamine, and serum, maintained maximal virus production. A final concentration of 1.5% serum in the total volume was preferable to 0.3% because of more consistent results. In most experiments, however, the lesser amount of serum was just as satisfactory. In small-scale production, where the supply of serum, vitamins, and glutamine are not limiting factors, the use of 150 ml of complete medium will increase total virus yield by eliciting a larger number of PFU per cell (Table 6).

Large-scale virus production. The foregoing experiments established the following standard virus-production procedures in 2-liter Baxter bottles. (i) For 57 cultures, 6-day-

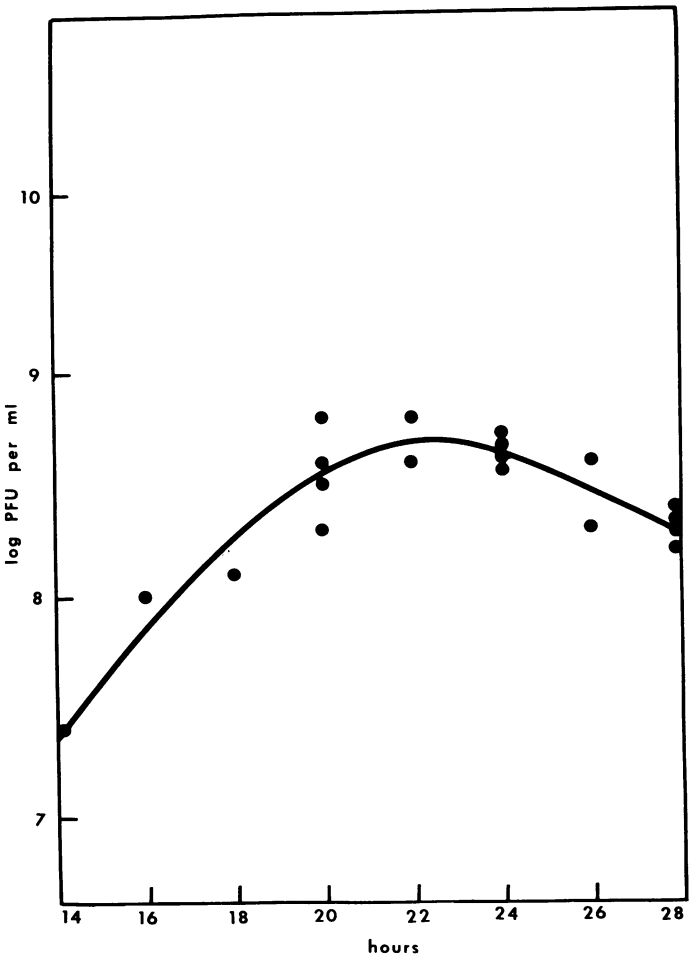

FIG. 3. *Yields of FMDV type A at different incubation times.*

TABLE 5. *Effect of culture age and number of cells on virus production*

Expt	Days	No. of cells × 10^7	Log PFU per ml	PFU per cell
1	3	6.6	7.3	14
	4	17.3	8.1	38
	5	38.2	8.2	22
	6	44.1	8.6	45*
2	3	30.6	6.3	0.3
	4	46.0	7.5	4
	5	56.1	7.9	7
	6	73.2	8.7	33*
3	4	43.6	8.5	37
	5	66.2	8.3	17
	6	74.1	8.6	28*

* Primary bovine kidney culture cells gave similar values for virus yield.

TABLE 6. *Reduction of serum, vitamins, and glutamine during virus production*

Virus production medium*			Virus yield	
First 30 min, 15 ml	Medium added after 30 min	Final vol	Log PFU per ml	PFU per cell
		ml		
Complete	Complete	50	8.5†	57
Complete	Complete	150	8.3	75
Complete	1% S	50	8.5†	55
Complete	1% S	150	8.0	52
Complete	No S, vit, or glu	50	8.5	48
5% S	No S, vit, or glu	50	8.5	59
1% S	No S, vit, or glu	50	8.5†	54
No S, vit, or glu	No S, vit, or glu	50	8.0	9

* Complete medium consisted of equal volumes of cell growth medium (GM) and 0.16 M tris with 10% serum. S, serum; vit, vitamins; glu, glutamine. Deleted serum was replaced by serum-free GM.

† Same virus titers produced in absence of vitamins and glutamine.

old BHK cell cultures grown without medium change in 57 Baxter bottles were infected with 15 ml of viral inoculum in GM-tris containing 5% calf serum. The multiplicity of infection was 0.01 to 0.05 PFU per cell. After 30 min, 35 ml of GM-tris without serum were added. The newly synthesized virus was harvested 24 hr later. (ii) For 190 cultures: it was not feasible to handle this number of cultures twice at the beginning of infection, as was required for maximal virus yield. Therefore, the cultures were infected with virus in 40 ml of GM-tris containing 1.5% serum. No further additions were made, and the virus was harvested after the time periods given below.

Collection of virus for purification. Fluids from infected cultures were harvested at 20 to 24 hr for type A virus, and at 18 hr for type C virus, into a chilled 5-gal carboy (Fig. 2): 2,800 ml from 57 cultures and 7,600 ml from 190 cultures. Although 18 hr was optimal for harvesting type C virus in these experiments, further passage adaptation yielded 14-hr harvests which contained as much infectious virus as those at 18 hr.

Purification of virus. After cooling below 0 C, the virus was concentrated and purified by stages of alcohol precipitation, extraction with organic solvents, density-gradient centrifugation in CsCl, and organic interface centrifugation as described in detail elsewhere (Bachrach et al., 1964). Table 7 lists parameters of virus concentration mass and purity for FMDV types A and C. Masses were determined by use of the extinction coefficient, 77.7, of virus at 259 mμ uncorrected for its light scattering. Alcohol precipitation effected large concentrations of virus with little loss of infectivity, as contrasted to extractions with *n*-butanol, $CHCl_3$, and Cl_2FCClF_2, which resulted in less than twofold concentrations as well as two- to threefold losses of virus. The lower recovery of virus was apparently caused by the passage of virus-containing aqueous solvent into the organic phase, since absolute infectivity values of the soluble portions of an alcohol precipitate and the aqueous phase from organic extractions in any experiment were nearly identical. The organic solvent treatment is, however, required to remove lipids and proteins which otherwise interfere with the first zoning run in CsCl (Bachrach et al., 1964). This run was accomplished in most cases without infectivity loss and with appreciable concentration and purification. Losses in this step never exceeded 50% except in experiment 8. The final organic interface run always concentrated the virus into 1.5 ml of light-scattering zones (LS_{org}), i.e., 0.5 ml in each of three SW 39 Spinco model L preparative centrifuge tubes. LS_{org} fraction infectivities ranged from 10.1 to 11.6 log PFU per ml; virus masses, from 0.4 to 17.0 mg. Overall infectivity recoveries ranged from 4 to 84%. The comparative constancy of the infectivity to mass values in the final column of Table 7 indicates that, with the exception of experiments 4 and 8, degradation of virus particles is not required for loss of infectivity. This is in accord with FMDV particle stability results presented elsewhere (Bachrach, 1964) which clearly show that FMDV at sodium ion strength 0.01 and pH 7.5 can be heated to 52 C without any degradation. Above this temperature, its ribonucleic acid (RNA) is first released and then denatured.

The present work confirms the earlier observation that virtually pure FMDV is obtained without a final flotation step (Bachrach et al., 1964). That is, type C virus in both runs of Table 7 gave a monodisperse 140*S* peak in the

TABLE 7. *Concentration and purification of FMDV from BHK cultures: infectivity, recoveries, and mass*

Expt	Type, strain	No. of cultures	Isolated fraction*										
			Harv	SP_{alc}		AqPh		LS_{sed}		LS_{org}			
			I	I	R	I	R	I	R	I	R	Amt	Log PFU per mg
												mg	
1	A, 119	57	8.5	9.6	56	9.6	34	—	—	11.0	11	3.1	10.5
2	A, 119	57	8.4	9.9	134	10.0	86	10.8	58	11.6	84	7.6	10.7
3	A, 119	57	8.6	9.6	46	9.5	20	10.5	21	10.6	8	0.9	10.7
4	A, 119	190	7.2†	8.7	91	8.8	50	9.4	21	9.5	5	0.4	9.9
5	A, 119	190	8.4	9.8	66	9.8	25	10.6	22	11.5	25	8.5	10.6
6	A, 119	190	8.2	9.4	24	9.7	30	10.6	29	11.4	27	9.0	10.4
7	A, 119	190	8.1	—	—	9.1	16	10.5	26	11.3	35	8.0	10.4
8	A, 119	171	8.1	—	—	9.1	31	9.7	4	10.1	3	6.5	9.3
9	A, 119	190	7.9	9.8	168	9.7	60	10.3	40	11.1	32	7.4	10.2
10	A, 119	190	8.5	9.9	50	9.9	30	10.8	34	11.3	15	17.0	10.1
11	A, 119	190	7.9	9.7	144	9.5	39	10.5	60	10.9	18	15.3	10.7
12	C, 3-Canefa	190	8.7	10.2	58	10.2	34	10.8	18	11.3	12	12.2	10.2
13	C, 3-Canefa	190	9.2	11.5	38	10.4	16	11.4	23	11.5	4	15.5	10.3

* Harv, harvested infectious fluid; SP_{alc}, soluble portions of alcohol precipitate; AqPh, aqueous phase from organic extractions; LS_{sed}, light-scattering zone from CsCl density-gradient centrifugation; LS_{org}, light-scattering zone from organic interface centrifugation; I, log PFU/ml; R, per cent recovery of PFU based on original harvest virus.

† Significantly lower infectivity caused by tenfold lower multiplicity of infection, i.e., 0.002, without lengthening virus growth period.

analytical ultracentrifuge, except for 10% of a 14*S* peak and its associated RNA. These smaller components are break-down subunits of FMDV. Additional criteria of the purity of virus are that the specific infectivities (Table 7, final column) were as high as those achieved previously and absorbance-temperature profiles were identical to those for FMDV (Bachrach et al., 1964; Bachrach, 1964).

DISCUSSION

BHK cells have been propagated in this laboratory for more than 60 passages with no apparent alterations in cell properties. Such properties as the rate of cellular growth and the extent of FMDV production were the same as in cell cultures started up from early passage frozen stock. The use of rotating 2-liter round bottles and modifications made in growth medium, including replacement of bicarbonate with tris buffer and of purified amino acids with lactalbumin hydrolysate fortified with histidine, have made the large-scale culture of BHK cells practical and economical. Handling of cultures is minimal, since the medium has sufficient nutrients and buffering capacity for the entire 6- to 7-day growth cycle of the cells.

It was found that BHK cells in Baxter bottles grow to five- to tenfold greater quantities than do primary bovine kidney cells over the 6-day period. Since approximately equal numbers of infectious particles are elicited by both cell types, a significant increase in virus production is obtained from BHK cultures, which is directly proportional to its greater cell numbers.

It is shown that the purification procedure developed for lower concentrations of FMDV (Bachrach et al., 1964) is applicable to masses of virus up to 17 mg. However, at this higher mass level, with the use of SW 25 and 37 rotors, the concentration of FMDV in the tightly packed light-scattering zones appears to exceed its solubility, and virus precipitates out of solution as a white amorphous solid. Even so, when dialyzed at 4 C against 0.05 M sodium phosphate buffer at pH 7.5, the virus remains physically intact for at least 2 months.

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