may account for the differences in findings from our acute studies measuring duodenal transport.

Summary. The effect of thyrocalcitonin on calcium transport in the rat duodenum was investigated in acute studies by an *in vivo* perfusion method. Absorption and lumen-to-plasma flux were slightly decreased in the thyrocalcitonin treated animals, but the differences were not statistically significant.

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Growth of Rubella Virus in BHK21 Cells. I. Production, Assay, and Adaptation of Virus.* (32283)

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The demonstration(1) that rubella virus (RV) grows in baby hamster kidney (BHK21) cells has been followed by the successful application of this system to virus assay(1), purification(2,3), the production of complement-fixing antigen(4), and the development of RV hemagglutinin(5). The principal advantages of using BHK21 cells in rubella virus work have been the high titers developed in this cell-type, and the feasibility of preparing large volumes of virus by infection of suspended cells. An additional advantage has been the development of a sensitive plaque assay made possible by the cytopathogenicity of RV in the BHK21 cells.

This paper describes the basic methodology of RV studies using BHK21 cells.

Materials and methods. Media. Stock cultures of the 2 cell lines were propagated at 36.5°C in monolayer in BHK21 medium which is based on Eagle's basal medium but contains double-strength amino acids and vitamins, and 0.1 mg/l of $Fe_2NO_3 \cdot 9H_2O$, 4.5 g/l of d-glucose, supplemented with 10% tryptose phosphate broth (Difco) and 10% heat-inactivated (56°C, 30 min) fetal calf serum(6). All media contained 100 IU/ml of penicillin-G, 50 μg/ml of streptomycin sulfate and 25 IU/ml of nystatin. The stock cultures were split twice a week in a ratio of 1:6 to 1:8 using as the cell-dispersing agent a mixture of 0.25% trypsin and 0.01% versene in Dulbecco's phosphate buffered saline (PBS) without Ca++ and Mg++.

Cell lines. Two lines of BHK21(6) were used in these studies. The first, 13S, is a rapidly growing cell derived from Stoker and MacPherson's Clone 13. Morphologically these cells are short fibroblasts. Although 13S grows on glass, its adherence to glass is poor and it does not form a confluent monolayer. Rather, growth proceeds by clumping of cells and subsequent release of clumped cells into the medium. Monolayer-grown 13S cells readily grew in suspension culture in the growth medium described above without adaptation. An increase in cell density from 5 $\times$ 10^5 to 1.5-2.0 $\times$ 10^6 cells/ml took place in 1.5-2 days.

The second cell line, WI-2, was derived as a clone from a population of BHK21 cells by the method of MacPherson and Montagnier (7). The WI-2 line morphologically consists

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of elongated fibroblastic cells which form good, well-maintained monolayers, and can be used for assay of infectivity. The fetal calf sera used in growth media had to be screened before use, since some sera tended to promote clumping of WI-2 cells on the glass.

Virus. Stocks, passages and assays. The RA 27/3 strain of RV(8) was employed in most of these studies. It was isolated in a kidney explant culture derived from an aborted human embryo and was passaged 4 times in WI-38 cells(9) before propagation in BHK21 cells. Beyond an initial passage in monolayer culture of WI-2 cells, all subsequent passages were done in suspension cultures of 13S cells by exposing the cells to a multiplicity *(M)* of 0.1-10 plaque-forming units (PFU). Supernatant virus was obtained by centrifuging suspended cells. The virus was stored at —70°C as stock harvests. Cell-associated RV was determined by counting and washing samples of cells 3 times with PBS, resuspending them in the original amount of maintenance medium, and then subjecting them to 3 cycles of rapid freezing and thawing. The RV interference titrations were done in cultures of primary African green monkey kidney (AGMK) cells according to the method of Parkman *et al*(10) and the titers were expressed as $IntD_{50}$/ml. For methods of RV plaque assays and end-point titration in WI-2 cells, see the experimental results.

Virus identification and sterility tests. The identity of the RA27/3 strain as RV was periodically confirmed during the BHK21 passages with neutralization tests performed in WI-2 or AGMK cells. We used pairs of acute and convalescent human sera from clinical rubella cases or rabbit hyperimmune serum prepared against another RV strain. No mycoplasma colonies developed within 7 days observation on Hayflick's(11) PPLO nutrient agar from samples taken from the stock WI-2, 13S cell cultures, and the RV harvest at each BHK21 passage level.

Sucrose gradient centrifugation. Five-ml "Lusteroid" tubes in the SW39 rotor of the Spinco Model L were used. The sucrose solutions used for gradients were buffered with Tris-EDTA. Preformed gradients were run at +4°C for 10-16 hours at 35,000 rpm and the rotor was brought to rest without braking. The fractions were collected in drops from a hole punctured in the tube bottom. Their densities were determined refractometrically in a Bausch and Lomb Abbe 3-L refractometer.

Experimental. Optimal conditions for virus production. Both the 13S and WI-2 clones were employed for preparation of high titer RV. For large-scale production of RV, suspension culture was the most practical. BHK21/13S cells, either grown in suspension or freshly dispersed after growth on glass, were infected with RV using an adsorption period of 120 minutes at 35°C. The cells at initial concentrations of 5-10 $\times$ 10^5 per ml were then maintained in BHK21 medium supplemented with 10% tryptose phosphate broth and only 5% serum. Our experience agrees with the report by Parkman *et al*(12) that many lots of postnatal calf sera contain heat-stable RV inhibitors. Each batch of serum, therefore, had to be pretested for suitability in RV studies. We had little difficulty, however, with fetal or Micro-Gamma® (Microbiological Associates) calf serum.

A magnetically driven stirring bar in an ordinary Erlenmeyer flask with a side arm for pressure release was fully adequate for keeping the cells in suspension. A temperature of 35°C was found to be preferable for incubation, since at this temperature slightly higher (up to 0.5 log/ml) RV titers were achieved than at 37°C. Owing to the high level of acid production by normal BHK21 cells and since a further enhancement of glycolysis is associated with RV infection in these cells(13), pH control was difficult. Furthermore, because RV is acid labile(12), it was necessary to keep the pH at or above 6.8. To do this, either filtered air was bubbled through cultures supplemented with 0.02% of Dow Corning Antifoam B Emulsion, or appropriate amounts of $NaHCO_3$ were added daily. With these techniques, we repeatedly obtained virus titers around 10^7-$10^{7.5}$ PFU/ml in supernatant culture fluids in 50-70 hours, provided a high enough multiplicity ($M>1$) was used to infect the cells.

Monolayer cultures of WI-2 cells maintained with Eagle's minimal essential medium

(MEM) plus 2% calf serum also yielded titers around 10^7 PFU/ml in the supernatant fluid after a high multiplicity of infection.

Quantitative analysis of virus growth. A typical one-step growth curve of the multiplication of high BHK21-passage RV in 13S suspension culture has been published(1). The first increase in cell-associated virus occurred around 12 hours. A further 4 hours elapsed before new extracellular virus appeared. At 24 hours the cell-associated virus titer reached its peak at the level of 2-3 PFU/cell. A steady state of virus production continued, and the extracellular titer achieved its peak of 30 PFU/cell in 64 hours. Infectious center studies at the peak of infection showed that more than half the cells were shedding RV.

Concentration and purification of supernatants from infected BHK21/13S cultures. Suspension cultures of BHK21/13 S cells (2-3 liters) at densities of 5-10 $\times$ 10^5 cells/ml were infected with RV (RA27/3 strain) at an input multiplicity of >1 PFU per cell. Fifty to seventy hours after infection the tissue culture fluids were centrifuged to remove the cells. The RV-containing supernatants were harvested, and their pH brought to 7.5-7.6 with the addition of 5.6% $NaHCO_3$. One-fiftieth volume of 1 M zinc acetate was then added slowly at +4°C, while the solution was constantly stirred by a magnet. A precipitate was formed within 60 minutes of standing and was collected by centrifugation. The dense sediment was redissolved in saturated EDTA solution buffered with Tris to pH 7.6 and was dialyzed in the cold for 24 hours against about 50 volumes of 0.01 M Tris-HCl buffer, pH 7.3. The dialyzing buffer was changed at least once. A 50-80% recovery of infectivity was obtained in the resuspended and dialyzed precipitate.

If the medium contained even trace amounts of *fetal* calf serum from the virus inoculum, the Zn^{++}-precipitation of RV was unsuccessful. Instead of the usual floccular precipitate a quite stable colloid was formed, from which RV could not be sedimented. Therefore only postnatal, complete or "agamma" (Micro-Gamma®, Microbiological Associates) calf serum was employed in connection with the zinc acetate precipitation method.

A large proportion of the host-cell debris and only about 10% of the infectivity was lost in centrifugation at 10,000 *g* for 10 minutes. After the medium-speed centrifugation the supernatants were run at 32,000 *g* for 150 minutes. For recovery of infectivity from the pellet the following method was chosen from among several tested. To consistently recover infectivity, it was necessary to resuspend the sediment in Tris-EDTA with the aid of a 24-gauge needle and a small syringe, followed by sonication for 2-5 minutes at 1.0 ampere in a Raytheon Magnetostrictive Sonic Oscillator, Model DF 101.

Density gradient centrifugation. The volume of the pellets resuspended after high-speed centrifugation was reduced to about 0.5 ml by forced dialysis against Carbowax 20-M. The preparation was subsequently layered on top of a 4.5 ml linear 25-55% sucrose gradient in Tris-EDTA. After centrifugation essentially all RV infectivity was recovered in a single peak which banded at a density of 1.19 g/ml (Fig. 1). Using the methods described above, 9% of the original virus was recovered in the peak fraction, which had a titer of 4.5 $\times$ 10^9 PFU/ml. The protein concentration had been decreased to 130 μg/ml from 4000 μg/ml.

Carrier state cultures. RV-infected BHK21 cells remained largely viable. In infected (M = 5-10) WI-2 monolayer cultures

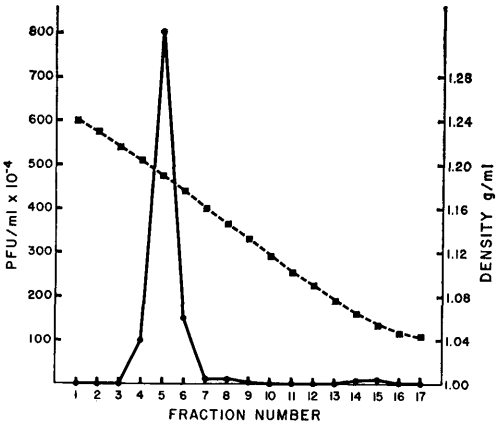

FIG. 1. Sucrose gradient centrifugation of supernatant fluids containing the RA27/3 strain of rubella virus propagated in BHK21/13S cells.

TABLE I. Effect of Maintenance Medium on Cytopathic Effect of RV in BHK21/WI-2 Cells.

Medium	Concentration of fetal calf serum (%)	Titer as $\log_{10} TCD_{50}$/ml	Duration of good control monolayers (days)
MEM	1	7.0	9
MEM	2	7.0	9
MEM	5	6.5	7
MEM	10	4.5	6
199	2	6.0	9
BHK21	0	5.0	7
BHK21	10	2.5	9
MEM + 10% TPB*	10	6.9	9
MEM + 1 g/l glucose	2	7.0	11

* Tryptose phosphate broth.

maintained with MEM + 2% serum, about 90% of the detached cells on day 4 and about 80% on day 5 excluded trypan blue. In RV-infected 13S cultures suspended in growth medium with 5% serum, close to 100% of cells remained viable although the cells divided at a slower rate. Extracellular virus titers approaching 10^7 PFU/ml were maintained in such chronically infected cultures for 40 days when the medium was changed every 4 to 5 days. The RV-CF-antigen titers obtained in these cultures are reported separately(14).

Titration of RV in BHK21 cells by cytopathic effect. RV produced a cytopathic effect (CPE) in monolayer cultures of WI-2 or 13S cells. The morphological changes were restricted to those of cell shape, size and attachment to the growth surface. No intracellular changes were detected. The CPE was first seen as an increased cellular refractility and rounding of infected cells. Decrease in size of cells, and detachment occurred later. At $M = 5\text{-}10$ the first changes were seen by 48 hours, and they became generalized within the next 24 hours.

The CPE of RV in WI-2 cultures was affected by various factors. At 37°C the CPE was somewhat slower and titrations more difficult to read than at 35°C due to a shortened maintenance of adequate control monolayers. Increased acidity, such as that rapidly produced in stoppered BHK21/WI-2 cell cultures, inhibited CPE. The titrations, therefore, were performed in Petri dishes incubated in an atmosphere of 3% CO_2 in air.

The effect of the composition of maintenance media on RV titrations is shown in Table I. MEM was preferable to BHK21 medium, and 2% serum was more effective than higher concentrations. The addition of 1 g/l of extra glucose to the MEM increased cell maintenance.

Although an extensive CPE also accompanied RV infection of 13S monolayers, they were not usable for end-point titrations due to their inferior maintenance of attachment to surfaces. Not all clones of BHK21 tested by us were equally susceptible to CPE when infected with RV. Clone 13 cells obtained from the American Type Culture Collection and an epithelioid variant(15) of BHK21 cells were much less affected by RV than was clone WI-2.

Plaquing. Our original plaque procedure(1) was modified. For optimum plaques, 4×10^6 WI-2 cells suspended in growth medium were seeded into 50-mm Petri dishes. After 24 hours at 36.5°C the monolayers were almost confluent. The growth medium was then removed and 0.2 ml of RV dilutions prepared in MEM + 2% calf serum added. After allowing a period of 120 minutes at 35°C for adsorption of RV, 5 ml of an overlay consisting of MEM, 2% inactivated fetal calf serum and 0.75% of Hercules 4-MH carboxymethylcellulose (gift of Hercules Powder Co., Wilmington, Del.) were added to each plate. After receiving the overlay the plates were immobilized for 6-7 days at 35°C in an atmosphere of 4% CO_2 in air. The semifluid overlay was then removed, and the plates washed briefly with water and stained with 0.25% crystal violet. This stain was left on the cells for about 15 minutes, after which the plates were rinsed with water. The plaques appeared as distinct areas 2-3 mm in diameter, containing few cells. In addition to 0.75% CMC, we used 0.4% agar (Noble's agar, Difco) or 0.2% agarose (Seakem® Agarose, Bausch & Lomb), but the quality of plaques was not as good, either when stained with neutral red or crystal violet.

The rate of adsorption of plaque-forming virus was studied by experiments in which

TABLE II. Adsorption of RV to BHK21/WI-2 Monolayers at 35°C.

Time (min)	Plaque count compared to value obtained after 180 min adsorption (%)
5	14
10	25
30	42
45	59
60	80
90	92
120	100

replicate monolayers were washed 3 times and overlaid at varying intervals after inoculation with PBS. Average data shown in Table II indicate that 80% of the plaque count obtained after 180 minutes of adsorption was reached in 60 minutes, and virtually 100% in 120 minutes.

Neutralization tests. The cytopathic effect in BHK21/WI-2 cells, either under liquid or under CMC overlays (plaques), was employed in tests for antibody to RV. After incubation at 37°C for 60 min, virus-serum dilution mixtures were assayed for residual infectious RV. It will be seen (Table III) that the titers obtained in WI-2 cultures either by the CPE-inhibition method or the plaque reduction test compared favorably to those obtained with the interference inhibition technique in AGMK. Moreover, the results were available somewhat earlier.

Adaptation of RV and cytopathogenicity of different strains to BHK21 cells. In the present study the RA27/3 strain of RV, previously propagated *in vitro* in WI-38 cells only, was repeatedly passaged in BHK21/13S cell cultures. At various passage levels the virus was titrated by plaque formation in BHK21/WI-2 cells and by the interference technique in AGMK cells. The results, compiled in Table IV, show that at low-passage (0-5) the RA27/3 strain titered equally in WI-2 and AGMK cells. During the 16 subsequent passages there was a progressive "deadaptation" of growth in AGMK cells, demonstrated by a loss of infectivity for AGMK cells in the standard ECHO 11 virus interference test. In contrast, plaque-forming ability in BHK21/WI-2 cells, and peak titers obtained in tissue culture fluid of BHK21/13S suspension culture after high multiplicity of infection, did not alter appreciably during the passages.

Discussion. In primary cells, RV does not usually produce a CPE(12). On the other hand, a variety of cell clones show CPE when infected with rubella. In several continuous

TABLE III. Comparative Tests for RV Neutralizing Antibody in BHK21/WI-2 and in AGMK Cells.

Cell and type of assay	Virus dose (log_{10})	Day of reading	Neutralizing antibody titer* Acute human serum	Neutralizing antibody titer* Convalescent serum
BHK21/WI-2 plaquing	1.7 PFU	6	<4†	32†
BHK21/WI-2‡ CPE	4.0 TCD_{50}	4	<4	16
		5	<4	8
		6	<4	8
	3.0 TCD_{50}	5	<4	16
		6	<4	16
	2.0 TCD_{50}	5	<4	64
		6	<4	32
	1.0 TCD_{50}	6	<4	64
AGMK interference§	4.0 $IntD_{50}$	10	<4	4
	3.0 $IntD_{50}$	10	<4	8
	2.0 $IntD_{50}$	10	<4	16
	1.0 $IntD_{50}$	10	<4	32

* Reciprocal titer; the sera used in tests: left, serum obtained from a 2-year-old child; right, serum obtained from the same child 6 weeks later after inoculation with rubella virus.

† Fifty percent plaque reduction.

‡ Readings made by observation of CPE on day indicated. First appearance of CPE was 4 days for 10^4, 5 days for 10^3, and 6 days for 10^2 and 10 TCD_{50} of RV.

§ Challenged at 7 days with 10^4 TCD_{50} of ECHO 11 virus.

TABLE IV. Comparative Assay of RV (Strain RA27/3) at Different BHK21-Passage Levels in BHK21/WI-2 Cells by Plaque Formation and in AGMK Cells by Interference Against ECHO 11 Virus.

BHK21-passage level	Titer in WI-2, $\log_{10}$ PFU/ml	Morphology of plaques (day 6)	Titer in AGMK, $\log_{10}$ $IntD_{50}$/ml	Titer in WI-2 $\log_{10}$ titer in AGMK
0	5.0	Clear, 2-3 mm	5.0	1
5	7.3	*Idem*	7.3	1
10	7.2	"	6.3	0.9
12	7.3	"	5.8	1.5
16	7.4	"	4.5	2.9
21	7.1	"	4.5	2.6

monkey kidney cell lines, a definite though slow CPE with intracytoplasmic inclusions is seen(16,17). In primary human amnion cells, a late and subtle CPE is produced, characterized by the aggregation of nuclear material and the presence of inclusion bodies(18). A more extensive CPE develops in primary human thyroid tissue cultures which, however, are too variable to make the system practical (19). Cultures of continuous lines of rabbit kidney(19,20), and rabbit cornea(21) demonstrate the most extensive CPE.

In comparison to the cytopathic effects seen in other cell lines, the CPE observed in BHK21 has the disadvantage of being markedly dependent on the conditions of incubation and on the maintenance of excellent uninfected monolayers. As applied to a plaque technique, however, the CPE is extremely useful for titration of BHK-grown viruses.

The principal use for BHK21 cells is in the production of high-titer virus, which makes possible procedures for purification of the virion(3), studies of host-cell metabolism under the influence of infection(13,22), and the elaboration of complement-fixing(4) and hemagglutinating antigens(5).

RV populations are modified by serial propagation *in vitro* in tissue cultures. High passage BHK21 virus was deadapted to growth in AGMK but not in RK_{13}. High passage RK_{13} virus grew well in BHK21. High passage AGMK virus, on the other hand, grew well in RK_{13} but not in BHK21.

Summary. Two clones of BHK21 baby hamster kidney fibroblasts were studied in relation to the growth of rubella virus (RV). One clone (13S) could easily be adapted to suspension culture. RV in titers of $10^{7.0}$-$10^{7.5}$ PFU/ml were found in supernatant fluids harvested between 50 and 70 hours. About 30 PFU per cell were produced in the first cycle of infection. Large quantities of virus could be produced in suspension culture, particularly when chronic infection was produced.

RV was cytopathogenic for BHK21 cells, and a second clone (WI-2) proved most useful in detection of this property. End-point titrations and neutralization tests could be performed in this cell strain, although environmental conditions had to be maintained within narrow limits. A plaque assay was developed using the WI-2 clone and a semisolid carboxymethylcellulose overlay. Small but distinct plaques appeared at 5-6 days.

During passage in BHK21/13S, the titer of RV measured by PFU in BHK21/WI-2 was constant, but the titer of RV measured by interference in African green monkey kidney cells gradually declined, indicating a considerable degree of deadaptation. Of the RV strains studied, those propagated in BHK21, WI-38 or RK_{13} cells produced larger and more numerous plaques in WI-2 cells than the two RV strains derived from monkey kidney cells.

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Growth of Rubella Virus in BHK21 Cells. II. Enhancing Effect of DEAE-Dextran, Semicarbazide and Low Doses of Metabolic Inhibitors.* (32284)

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The enhancement of virus growth in animal cell cultures by changes in environmental conditions, by various chemical substances, by physical treatment and by "helper" viruses, has been actively studied recently. Such enhancement phenomena, which have obvious bearing on the control mechanisms of viral multiplication, have also been of practical significance in the laboratory investigation of several viruses. The foregoing report(1) described conditions for the study of rubella virus (RV) in tissue cultures of the WI-2 and 13S clones of baby hamster kidney BHK21 cells. In this paper we show that RV growth and study may be further improved, even in these highly susceptible cell systems, by addition of diethylaminoethyl-dextran (DEAE-dextran) and semicarbazide (SCZ) and to some degree also by small doses of Actinomycin D (ACTD), 5-Fluoro-2'-Deoxyuridine (FUdR) and UV pretreatment of host cells. Experimentation with DEAE-dextran was prompted by the finding that it enhances the infectivity of poliovirus and poliovirus RNA(2,3), while studies of SCZ were stimulated by the work of Grossberg and Lwoff(4,5) on the exaltation of poliovirus replication.

Materials and methods. Tissue culture, media, virus stocks and assays were described in the previous paper(1). Abbreviations: CMC = carboxymethylcellulose, CPE = cytopathic effect, M = multiplicity of virus per cell (PFU/cell) in inoculum, MEM = Eagle's minimum essential medium, NDV = New Castle disease virus, PBS = Dulbecco's phosphate buffered saline, PFU = plaque forming unit(s).

Chemicals. DEAE-dextran: (diethylaminoethyl-dextran, Pharmacia), prepared from dextran with M.W. = 2×10^6. A stock

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