tion achieved in 7 to 10 days, in contrast with results using most defined media, (3) the growth of these cells in suspension culture, which is an ideal system for both nutritional and parasite-host cell relationship studies. The effectiveness of methylcellulose as a physical protective agent for cells grown in a simple, chemically defined medium has been demonstrated.

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Multiplication and Cytopathology of Coxsackie A-21 Virus in Rotated and Stationary Tissue Culture. (27465)

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Coxsackie A-21 virus (Coe) is an enterovirus which propagates and produces a cytopathic effect (CPE) in certain human continuous and primary embryonic tissue cultures (1,2,3,4). This agent was recently shown to be associated with acute undifferentiated upper respiratory illness in military personnel (5,6). During controlled epidemiologic studies which established the role of Coxsackie A-21 virus in respiratory disease it was observed that production of CPE by this virus was enhanced when inoculated HEp-2 tissue cultures were rotated during incubation. The quantitative aspects of virus replication and cytopathology as effected by rotation of cul-

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tures in a roller drum were investigated and the results form the basis of the present report.

Materials and methods. Tissue culture. Primary human embryo kidney (HEK) and HEp-2 cultures were purchased from Microbiological Associates, Inc. Prior to inoculation the HEp-2 cultures were washed twice with Hanks' balanced salt solution (BSS) following which 1.5 ml of Eagle's basal medium with 5% inactivated chick serum (56°C at 30 minutes) and 0.002 M glutamine was added. An antibiotic mixture was added to all media containing at final concentration: penicillin, 100 units per ml; streptomycin 100 gamma per ml; mycostatin 50 units per ml; and tetracycline 100 gamma per ml (SPAM). The maintenance medium was changed at 3-4-day intervals. Primary HEK cultures were maintained with medium 199 with 2% inactivated chick serum and 0.002 M glutamine, which was changed at 4-day intervals.

Tissue cultures were incubated at 34°C and observed daily or at 2-day intervals for at least 18 days. "Rotated" tubes were incubated on a revolving drum rotating 12 r.p.h. "Stationary" tissue cultures were incubated at a 5° angle.

Virus strains. The strains of Coxsackie A-21 virus employed in this study were recovered from the pharynx of patients during an outbreak of acute respiratory disease associated with this virus among military personnel at Camp Lejeune, N. C.(5,6). Original isolations were made from pharyngeal throat swabs as previously described. The prototype strain of this epidemic was designated as Bouma and has been shown to be serologically indistinguishable from the Coe strain of Coxsackie A-21 virus(7). Virus strains used in these experiments were identified by hemagglutination-inhibition. Each strain used had been previously successfully reisolated in either HEK or rotated HEp-2.

Comparison of rotated and stationary HEp-2 cultures for recovery of virus from throat specimens. Two-tenth and 0.4 ml of original throat-swab material were inoculated into each of two HEp-2 cultures. One pair of culture tubes (0.2, 0.4) was incubated on a roller drum, and the other pair stationary, and each pair was observed daily for CPE.

Infectivity titrations of throat specimens. Ten-fold dilutions of virus were made with Hanks' BSS. Two-tenths ml of each dilution was inoculated into 4 to 10 cultures of HEp-2 and HEK. Half of the tubes at each dilution were incubated on the rotating drum and half stationary. Infectivity titers based upon CPE were calculated by the method of Reed and Muench(8).

Quantitation of virus production in rotated and stationary HEp-2 cultures. An original throat-swab specimen was diluted to contain $10^{0.8}$ and $10^{2.2}$ TCD_{50} per 0.1 ml respectively of Coxsackie A-21 virus. Thirty HEp-2 cultures were inoculated with 0.1 ml of fluid at each dilution. One-half of the cultures were incubated stationary and one-half were rotated. At 4 hours the cell sheet was washed 3 times to reduce the quantity of unabsorbed virus. Supernatant fluid and cells from 2 tubes of each dilution were harvested separately at 5 hours, 1,2,3,4,6,8, and 12 days post-inoculation. The amount of virus present in these samples was determined by titration using rotated HEp-2 cultures.

Results. Sensitivity of rotated and stationary HEp-2 cultures to Coxsackie A-21 virus. Throat-swab fluids from 49 Coxsackie A-21 positive specimens, selected at random, were reinoculated into HEp-2 cultures, one-half of which were incubated on a rotating drum, and one-half stationary. As shown in Table I, rotated HEp-2 cultures were more sensitive for virus isolation. A cytopathic effect occurred more rapidly in rotated HEp-2 cultures. Forty-three of 49 Coxsackie A-21 strains were positive in rotated HEp-2 by 4 days. In stationary cultures the majority of specimens were not positive until 11 days after inoculation. A blind passage was performed at 15 days from the 11 negative stationary cultures. Five were positive when passage was made into rotated cultures; 4 of these 5 were also positive when passed into stationary cultures.

The effect of rotation upon the sensitivity of HEp-2 cells to naturally occurring Coxsackie A-21 virus was further studied by performing simultaneous titrations of throat-swab specimens in rotated and stationary cul-

TABLE I. Time Required to Recognize Cytopathic Effects Produced by Naturally Occurring Coxsackie A-21 Virus when Tissue Cultures Rotated or Kept Stationary.

Day	No. of isolates which produced a 50% cell destructive effect on indicated day	
	Rotated HEp-2	Stationary HEp-2
1	1	
2	8	
3	24	
4	10	
5	4	
6	1	
7	1	3
8		2
9		1
10		
11		25
12		1
13		2
14		
>15		4
Total	49	38

No. not isolated in stationary HEp-2 = 11

Specimens were inoculated into four HEp-2 cultures, 2 of which were rotated and 2 held stationary.

tures. HEK was used as a comparative tissue culture system. Titers of virus determined in rotated HEp-2 cultures were generally 10- to 1000-fold greater than those detected in stationary cultures (Table II). For 5 of these specimens the infectivity titer in stationary cultures was less than 10 TCD_{50} per ml. In addition, the final titer was in each instance attained earlier in rotated cultures. In HEK no consistent trend favoring either rotation or stationary incubation was observed. Using either manner of incubation maximum titers were demonstrable more rapidly in HEK than in rotated HEp-2 cultures.

Virus multiplication in rotated and stationary cultures. The quantitative aspects of virus multiplication in stationary HEp-2 cultures without demonstrable CPE was compared with that occurring in rotated HEp-2 tissue cultures. HEp-2 roller tubes were inoculated with $10^{0.8}$ or $10^{2.2}$ TCD_{50} of virus from a throat-swab specimen. Subsequently the supernatant fluid and the cell sheet were titrated separately at 5 hours, 1, 2, 4, 6, 8, and 12 days post-inoculation. The results are shown in Fig. 1. No CPE was demonstrable in stationary HEp-2 inoculated with

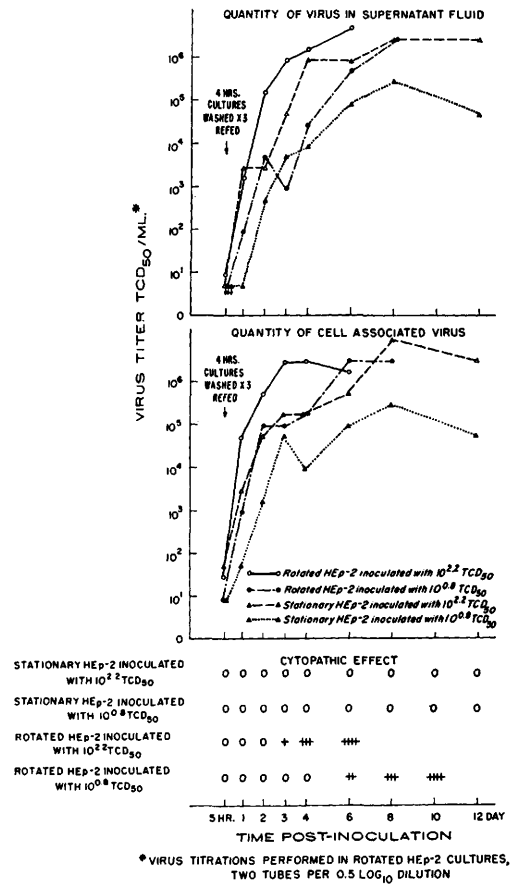

FIG. 1. Comparison of virus production and cytopathic effect in rotated and stationary HEp-2 cultures inoculated with naturally occurring Coxsackie A-21 virus.

either virus dose during a 12-day observation period. In rotated cultures inoculated with $10^{2.2}$ TCD_{50} CPE was first evident at 3 days, and progressed to a maximum at 6 days. Rotated cultures receiving the lower dose showed minimal CPE at 6 days and complete CPE by 10 days. Although it appeared that virus multiplication proceeded more rapidly in rotated cultures, the amounts of virus produced in both types of culture were similar. It is of interest that cytopathic effects were not observed in stationary cultures despite the production of such large quantities of virus. It is probable that factors in addition to or other than the quantity of virus produced within the HEp-2 determine the occurrence of Coxsackie A-21 cytopathology.

Induction of CPE by rotation. As shown

TABLE II. Sensitivity of Stationary and Rotated Cultures to Naturally Occurring Coxsackie A-21 Virus.

Specimen	Incubation of cultures	Titer (TCD_{50}/ml log_{10}) of virus in throat specimen at indicated time after inoculation as determined in* HEp-2 Day 2	HEp-2 4	HEp-2 6	HEp-2 8	HEp-2 12	HEp-2 15	HEK Day 2	HEK 4	HEK 6	HEK 15
1	Stationary	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	3.2	3.7	
	Rotated	<1.0	3.2	3.2	4.2	———	———	3.7	———	———	———
2	Stationary	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	1.7	5.2	———	———
	Rotated	<1.0	2.7	2.7	3.2	———	———	2.7	4.2	———	———
3	Stationary	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	1.7	———	———	———
	Rotated	<1.0	1.7	3.2	———	———	———	2.2	3.2	3.7	——
4	Stationary	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	2.2	3.2	——
	Rotated	<1.0	<1.0	2.2	2.2	3.2		3.2	———	———	———
5	Stationary	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	2.2	3.2	4.2	——
	Rotated	<1.0	<1.0	2.2	———	———	———	3.2	4.2	———	———
6	Stationary	<1.0	<1.0	<1.0	<1.0	<1.0	1.7	<1.0	3.2	———	———
	Rotated	<1.0	2.2	———	———	———	———	2.7	———	———	———
7	Stationary	<1.0	<1.0	<1.0	<1.0	2.2	——	2.7	4.7	———	———
	Rotated	<1.0	3.2	———	———	———	———	3.2	5.2	———	———
8	Stationary	<1.0	<1.0	<1.0	1.7	2.2	——	<1.0	2.2	———	———
	Rotated	<1.0	<1.0	3.2	———	———	———	2.2	3.2	———	———
9	Stationary	<1.0	<1.0	2.2	———	———	———	<1.0	3.2	4.2	——
	Rotated	<1.0	2.2	2.2	3.2	———	———	1.7	3.7	———	———
10	Stationary	<1.0	<1.0	<1.0	1.7	———	———	<1.0	3.2	———	———
	Rotated	<1.0	1.7	2.2	———	———	———	2.2	3.7	———	———

* 15-day observation period. ———, no further increase in titer.
Four cultures were used for each 10-fold dilution.

in Table III, CPE could be accelerated in stationary HEp-2 cultures by transferring them to the roller drum. For this experiment $10^{2.5}$ TCD_{50} of naturally occurring virus was inoculated into stationary cultures, and at 1-day intervals five tubes were transferred to the roller drum. In initially rotated cultures CPE was demonstrable by the first day and complete by the third day. Cultures maintained stationary throughout failed to show CPE until the 7th day, and this effect slowly progressed to a maximum at 13 days. In stationary cultures, which were negative when transferred to the rotating drum at day 1 or day 2, CPE was evident in each instance at day 4, and maximal by day 5. Cultures which

TABLE III. Effect of Rotating Coxsackie A-21 Infected HEp-2 Tissue Cultures Previously Held Stationary upon Development of Cytopathic Effect.

	Manner of incubation	Cytopathic effect on indicated day* 1	2	3	4	5	6	7	8	9	10	11	12	13
(1)	Rotated	1+†	2+	3+										
(2)	Stationary	0	0	0	0	0	0	1+	1+	1+	2+	2+	3+	4+
	Stationary→ Rotated after:													
(3)	Day 1	0	0	0	0 to 1+	2+ to 4+	3+							
(4)	" 2	0	0	0	0 to 1+	4+								
(5)	" 3	0	0	0	0 to 1+	4+								
(6)	" 4	0	0	0	0	1+ to 3+	4+							
(7)	" 5	0	0	0	0	0	2+ to 4+	4+						

* Each tube inoculated with throat specimen containing 320 TCD_{50} Coxsackie A-21 virus.
† 1+, less than 25% cell destruction; 2+, 50% cell destruction; 3+, 75% cell destruction; 4+, complete cell destruction.
Four cultures were used for each experimental group.

TABLE IV. Effect of Type of Incubation (Rotated or Stationary) upon Time Required for Development of Cytopathic Effect in Cultures Inoculated with Varying Quantities of Coxsackie A-21 Virus.

Inoculum* Log_{10} TCD_{50}	Days to 50% degeneration of cell sheet in Rotated HEp-2	Stationary HEp-2
4.8	2	2
3.8	2	3
2.8	2	8
1.8	3	9
.8	3	13

* Inoculum consisted of dilutions of a first HEp-2 passage of Coxsackie A-21 virus.

were negative when transferred at day 3, 4, and 5 demonstrated early CPE one day after being rotated and maximum CPE one day thereafter. The data indicate that although CPE was not observed after 1-3 days of stationary incubation certain changes occurred which permitted the induction of CPE when the cultures were rotated.

Effect of inoculum size. Evidence that the formation of CPE in stationary HEp-2 cultures was related in part to inoculum size is presented in Table IV. In order to utilize a large inoculum for this experiment, it was necessary to employ tissue culture passage material. An inoculum of $10^{4.8}$ TCD_{50} of first HEp-2 passage Coxsackie A-21 virus produced CPE in stationary HEp-2 in 2 days, similar to the incubation period in rolled HEp-2. The effect of rotation upon development of CPE became more marked as smaller quantities of virus were employed for initiation of infection.

Cytopathic effect. Differences in CPE between rotated and stationary HEp-2 cultures were also observed. In rotated cultures, the earliest evidence of CPE was the appearance of discrete large ovoid cells (av. dia. 35 μ) of varying shapes, found usually towards the cap end of the tube and at the edges of the cell sheet; many round cells (av. dia. 15 μ) were interspersed in the same areas. Both types of cells showed karyolysis. Cellular degeneration rapidly evolved towards the lower end of the tube, usually involving the entire cell sheet within 24 hours. In stationary HEp-2 cultures the first evidence of CPE was the appearance of abundant numbers of small round cells (av. dia. 15 μ) throughout the monolayer; distinctive large ovoid cells were not observed. Disruption of the cell sheet occurred within 24 hours after the onset of CPE, and numerous "tags" of 4 to 6 small round cells strung together were formed. These "tags" were adherent to the glass surface at one pole and floated freely in the medium at the opposite pole. Cell clusters detached from the glass surface as complete degeneration of the cell sheet occurred.

Discussion. It has been shown that the production of CPE observed in HEp-2 cells infected with naturally occurring Coxsackie A-21 virus is favored by rotation of the cultures during incubation. Rotated HEp-2 cultures were a more sensitive and rapid system for virus isolation than comparable stationary cultures. Thus it is advisable to employ rotated HEp-2 cultures in attempts to isolate Coxsackie A-21 virus from field specimens. Johnson, Bloom, *et al.*,(5,6) have reported the isolation of more than 250 strains of Coxsackie A-21 virus using rotated HEp-2 cultures during an outbreak of minor upper respiratory illness with which the virus was associated at Camp Lejeune, N. C.

To date rotation of continuous cell lines has not been used extensively in virus isolation attempts. Recently, however, rotation of cultures of primary cell lines has been employed advantageously for isolation and propagation of certain enterovirus-like agents (9), ECHO 28(10), and chick embryo-adapted poliovirus(11). Frothingham demonstrated that rotation favored production of CPE in 9-day-old human amnion cultures infected with chick embryo adapted poliovirus and that virus multiplication proceeded at a more rapid rate in these cultures than in stationary cultures; this effect was not observed with cultures inoculated when 30 days old. Similarly in our studies the rate of multiplication of Coxsackie A-21 was accelerated by rotation of HEp-2 cultures.

Although the mechanism whereby rotation enhances cytopathology of Coxsackie A-21 infection of HEp-2 is not known, certain factors can be eliminated from consideration: 1) the occurrence of cell damage is not dependent

upon the quantity of virus produced since equivalent amounts were detected in rotated and stationary cultures; 2) CPE is apparently not related to the rate of multiplication since stationary cultures infected with the higher multiplicity had a more rapid rate of virus multiplication than the low multiplicity rotated cultures, yet only the latter showed cell damage; 3) variation in the virus genome is unlikely since the same inoculum was used for rotated and stationary cultures. In addition it is improbable that variants with an increased cell destructive capacity were selected during initial passage in rotated cultures since CPE with Coxsackie A-21 virus from the tenth rotated culture passage was still favored by rotation.

It is of interest that Baron *et al.*(12,13), have demonstrated that an increase in available oxygen improved propagation of certain viruses in tissue culture, and that these findings were correlated with decreased viral inhibitory action of interferon. It is possible that the effect of rotation upon CPE in HEp-2 cells may be produced by an increase in oxygenation of the cells.

Additional evidence for the dissociation of production of large quantities of virus and demonstrable cytopathology was provided by the studies of Hirst with influenza A in mice (14). Unadapted influenza A introduced into the lungs of mice attained high titer with few or no demonstrable lung lesions or deaths from pneumonia. On serial passage of the virus in the lungs of mice, a mouse adapted variant was selected which produced lung lesions and deaths from pneumonia although it multiplied to a level no higher than the non-adapted strain. In this instance the cell destruction was related to a change in the genetic properties of the influenza A virus. However, in our studies the effect of rotation appears to be non-genetic.

Summary. Rotation of HEp-2 tissue cultures infected with Coxsackie A-21 virus enhanced the production and rapidity of appearance of CPE. In comparable stationary cultures the formation of CPE was delayed and often it was not demonstrable. Although the rate of virus replication was slightly faster in the rotated cultures the amounts of virus produced in both systems were similar.

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