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An Investigation of the Replication of Coronaviruses in Suspension Cultures of L132 Cells

By

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With 6 Figures

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Summary

A system is described for the study of the multiplication of two coronaviruses, both of human origin. Low efficiences of adsorption and infectious centre formation were observed. The viruses were found to have a 4—6 hour eclipse phase in L 132 cells at 33° C. Both viruses were completely inhibited by raising the incubation temperature to 39° C, and there was also a depression in the yield of virus at 30° C compared with control cultures maintained at 33° C. "Shift" experiments have indicated that the high temperature-sensitive event occurred fairly late on in the replicative cycle, but the time could not be accurately determined.

1. Introduction

The discovery of ether-labile viruses, serologically unrelated to myxoviruses, in cases of upper respiratory infection in man (Tyrrell and Bynoe, 1965; Hamre and Procknow, 1966) was the first stage in the recognition of a new group of viruses, found in infections of both man and animals. These viruses were named "coronaviruses" (Nature, 1968) because of their characteristic structure.

Coronavirus virions are pleomorphic, varying in diameter from 800 to 1500 Å (Almeida and Tyrrell, 1967). They are normally covered with a 150 Å-thick fringe of club-shaped projections; these are easily removed by either physical or chemical treatments.

Several viruses with this morphology have been isolated from man (*e.g.* Tyrrell, Bynoe and Hoorn, 1968; McIntosh *et al.*, 1967; Kapikian *et al.*, 1969). Other viruses of this group include infectious bronchitis virus of chickens (Berry *et al.*, 1964), murine hepatitis virus (Almeida and Tyrrell, 1967), and transmissible gastroenteritis virus of pigs (Tagima, 1970). Most of the human strains and several of the animal strains have been shown to be serologically inter-related (McIntosh *et al.*, 1969; Bradburne, 1970).

Apart from studies of the replication cycle and morphogenesis of mouse hepatitis virus (David-Ferreira and Manaker, 1965; Chany and Robbe-

Maridor, 1970) and of the comparative morphogenesis of a human coronavirus (229 E) and avian infectious bronchitis virus (Hamre, Kinding and Mann, 1967; Becker *et al.*, 1967), there are few reports on studies of coronavirus replication. These have awaited the development of a sensitive plaque assay (Bradburne and Tyrrell, 1969). The results reported here are derived from single-cycle growth curve experiments with two human coronaviruses in suspended tissue culture cells.

2. Materials and Methods

2.1. Viruses

229 E virus was received from Dr. D. Hamre and was passaged twice in human embryo lung, once in human embryo tracheal organ culture and three times in human volunteers (Bradburne, Bynoe and Tyrrell, 1967). The virus was recovered in tissue cultures of L 132 cells, from clinical specimens from these volunteers, and was passaged thereafter only in these cells.

LP virus (Tyrrell *et al.*, 1968) was recovered from a nasal washing taken from a volunteer who had been infected with organ culture fluid (3rd passage after the original isolation). It was passaged twice in L 132 cells, 3 times in human diploid lung cells and then 6 times in L 132 cells.

2.2. Tissue Cultures

L 132 cells (Davis, 1960) were grown and maintained as previously described (Bradburne and Tyrrell, 1969). For use in suspension cultures, cells were dispersed with trypsin-versene, washed, and suspended in Eagle's medium containing 2% foetal calf serum and 0.02 M HEPES buffer (Sigma). Cells were stirred magnetically at 300 r.p.m. for 12 hours at 33° C before use. Viable cell counts were made in a haemocytometer on the basis of their resistance to staining with 0.1% trypan blue.

2.3. Infectious Centre Assays

These were performed by diluting infected cells 100-fold in ice-cold medium; washing these cells three times in similar volumes of medium and then resuspending cells in warm medium. These cells were plated out, at various dilutions, onto drained monolayers of L 132 cells at 37° C and were allowed to attach for 60 minutes. The cell monolayers were overlaid with the standard medium for plaque production.

2.4. Single Cycle Growth Curve Experiments

Virus stocks were freed from macrocellular debris by centrifugation at 2500 *g* for 10 minutes. Virus was warmed to 37° C mixed with packed L 132 cells and stirred at 33° C for 20 minutes. After this period, cells were washed rapidly three times in ice-cold medium. Trial experiments showed that the final wash never contained more than 10^2 p.f.u./ml. The cells were counted and resuspended in medium at 33° C. Unless otherwise stated, an input multiplicity of between 4 and 10 p.f.u./cell was used. The start of the infectious cycle was taken as being half-way through the adsorption period.

Periodically, small samples were withdrawn from infected cell suspensions and either diluted 10-fold in a stabilizing diluent and frozen, or the cells spun out, washed and disrupted by freezing and thawing. All samples were stored at —70° C before titration.

3. Results

3.1. Efficiency of Formation of Infectious Centres

In preliminary experiments it was discovered that very little 229 E virus was adsorbed to L 132 cells suspended at 4° C. The efficiency of infection determined as the ratio of plaque-forming units of virus adsorbed per cell replicating infection

virus (infectious centre) was very low. Although no greater proportion of virus was adsorbed to cells suspended at 33° C, the number of infectious centres formed was considerably higher (Table 1).

Table 1. *The Formation of Infectious Centres by 229 E Virus in L 132 Cells at 4° and 33° C*
1.2×10^6 cells suspended in 6×10^6 p.f.u. of 229 E virus at the start of the adsorption periods

	Cell count/ml after adsorption	Virus titre/ml after adsorption	Adsorbed virus[a] p.f.u./cell	Infectious centres/ml	Per cent infected cells[b]	
					a	b
12 hours at 4° C	9.5×10^4	5.0×10^6	0.8	1.8×10^4	1.6	18.6
60 minutes at 33° C	8.1×10^5	5.7×10^6	0.2	2.2×10^5	18.0	27.2

[a] "adsorbed virus" were calculated on the cell concentration at the start of the experiment.
[b] a = % infected cells calculated on initial cell concentration.
b = % infected cells calculated on final cell concentration.

Table 2. *The Rate of Formation of Infectious Centres by 229 E Virus at Two Cell Concentrations at 33° C*

Time (min.)	Control virus p.f.u./ml[a]	Input Multiplicity			
		4.5 p.f.u./cell		0.9 p.f.u./cell	
		IC[b]/cell	Supernatant virus (p.f.u./ml)	IC/cell	Supernatant virus (p.f.u./ml)
0	9×10^6	0.00	9×10^6	0.00	9×10^6
2.5		0.092		0.0021	
5.0		0.130		0.0045	
7.5		0.140		0.0037	
10.0		0.093	6×10^6	0.0038	8.1×10^6
15.0		0.081		0.0043	
20.0	9×10^6	0.065	7.5×10^6	0.0042	4.3×10^6
40.0		0.090		0.0055	
60.0	7×10^6	0.114	5.0×10^6	0.0048	6.0×10^6
120		0.230		0.0055	
180	3×10^6	0.321	2.6×10^6	0.0062	4.0×10^6

Virus concentration $= 9 \times 10^6$ p.f.u./ml
Low cell concentration $= 2 \times 10^6$ cells/ml
High cell concentration $= 1 \times 10^7$ cells/ml

[a] "control virus" stirred in cell-free medium at 33° C.
[b] infectious centres.

The effect of varying the concentration of cells on the formation of infectious centres was studied. Cells were suspended in the same concentration of virus at two differing concentrations (Table 2). Frequent estimations of the proportions of infectious centres in each mixture were made during a total incubation period of 180 minutes.

The higher input multiplicity (*i.e.*, the more dilute cell suspension) produced between 2 and 5-fold more infectious centres at each sampling time. The infectious centre counts reached 30—50% of maximal levels within 15 minutes of mixing virus and cells, but the levels continued to rise throughout the incubation period.

Possible explanations of the inefficient infection of L 132 cells by 229 E virus were considered. Firstly, the L 132 cell cultures might be constituted of mixed populations of resistant and susceptible cells. Cloning experiments to confirm this were not practicable. Secondly, the pool of virus used to infect these cells could contain interferon or similar substances which would prevent either the formation or expression of infectious centres. Because of the large volumes of fluid used to wash infected cells, it seemed unlikely that interferon would be active in this

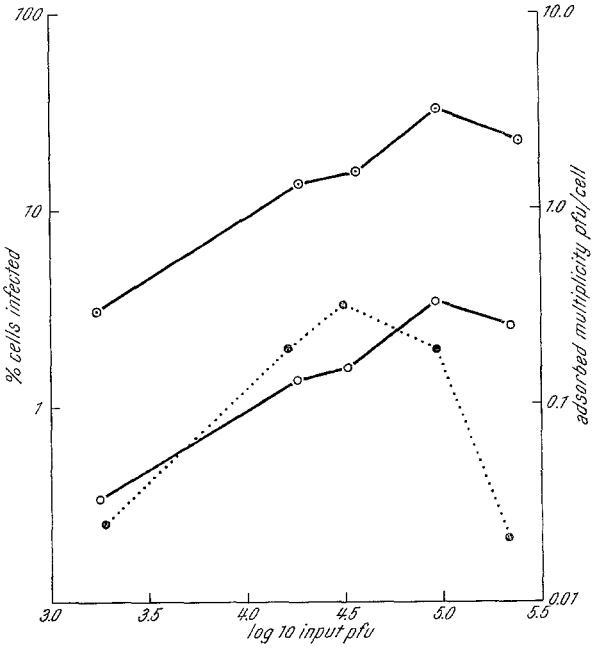

Fig. 1. The theoretical and observed relationships between the adsorbed multiplicity of 229 E virus on L 132 cells and the percentage of cells infected, at various dilutions of infecting virus
o ——— o adsorbed p.f.u./cell; ◉ ——— ◉ theoretical per cent infected cells; ● ····· ● actual per cent infected cells

system unless it had become fixed to the cells. To detect any interferon present in the virus pool, the infectivity was destroyed by dialysis against buffer at pH 2 for 12 hours at 4° C. After subsequent dialysis against pH 7.2 buffer, no interferon-like activity could be detected as judged by the lack of growth inhibition of Sindbis virus in L 132 cells. The third possibility was that some form of autointerference might be operating, as has been found with arboviruses or with vesicular stomatitis virus (CRICK, CARTWRIGHT and BROWN, 1969) under certain conditions. In conditions of autointerference, the normal relationship between the input multiplicity and the efficiency of infection or yield of virus does not operate. Thus, dilution of virus might result in an increased number of infected cells as the interfering activity is diluted out. The relationship between the dilution of a pool of 229 E virus and the ability of the pool to form infectious centres was therefore studied. The adsorption period was 2 hours at 30° C, after which cells were washed and assayed for infectious centres as described. The experiment shown in Figure 1 used a concentration of 1×10^6 cells per ml and the input multiplicity was varied

from 0.82 to 0.0082 p.f.u./ml. However, the proportion of infectious centres rose on dilution of the virus pool, reaching a maximum at a 1 in 5 dilution of virus. At a dilution of 1 in 100 the virus produced as many infectious centres as were formed using undiluted virus. Nevertheless, even the maximum number of infectious centre formed was 5-fold lower than the level predicted from the proportion of virus which had adsorbed to cells.

Table 3. *Concentrations of Viruses, Cells; and Other Data for the Growth Curves Shown in Figure 2*

	LP	229 E
Total number of cells	4.7×10^7	3.3×10^7
Virus input (total p.f.u.)	2.5×10^8	1.1×10^8
Input Multiplicity (p.f.u./cell)	5.3	3.3
Adsorbed Multiplicity (p.f.u./cell)	0.28	0.052
Total Infectious Centres (IC)	6.4×10^6	3.7×10^6
Percentage Infected	13.0	11.2
Intracellular virus titre (p.f.u./ml) at 10 hours	1.8×10^4	3.2×10^4
Extracellular virus titre (p.f.u./ml) at 10 hours	1.6×10^3	2.0×10^4
Maximum yield (p.f.u./IC)	1.3	2.1

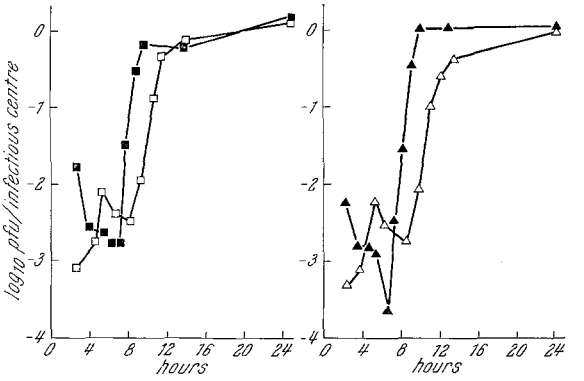

Fig. 2. The production of cell-associated and supernatant 229 E and LP viruses in L 132 cells at 33° C and pH 7.2 (see also Table 3)

■ ——— ■ 229 E cell-associated virus; □ ——— □ 229 E supernatant virus; ▲ ——— ▲ LP cell-associated virus; △ ——— △ LP supernatant virus

3.2. The Time-Sequence of Viral Replication in L 132 Cell Cultures

Using the standard conditions, detailed in the "Materials and Methods", the growth curves of both 229 E and LP viruses in L 132 cells were investigated. The production of cell-associated and cell-free virus was measured; infectious centres and adsorbed multiplicities were also estimated. The data for these experiments are given in Table 3 and the results displayed in Figure 2.

The "eclipse" phase (*i.e.* the interval between adsorption and detectable virus formation) was 4—7 hours for both viruses, and averaged about 4.5 hours. Because of the low proportion of cells initially infected, multiple cycles of infection were likely, but in fact did not seem to occur since virus titres reached a maximum at 10—12 hours and stayed at that level for the remaining 12 hours of the experiments. This only applied to the cell-associated virus; cell-free virus titres continued to rise throughout the period of observation. At 10 hours after infection there was 10-fold less cell-free than cell-associated virus; but with 229 E virus, the virus was released as rapidly as it was formed. In all cases the final yield of virus was very low (2—6 p.f.u./infectious centres).

3.3. The Effects of Temperature on Virus Replication

It has been shown (STOTT and HEATH, 1969) that rhinoviruses (the other main group of viruses implicated in upper respiratory tract disease in man) have their replication inhibited by temperatures above 39° C. This might be the result of the low physiological temperature of their natural environment, the nasal epithelium. Characteristically, both rhinoviruses and coronaviruses are more easily isolated in tissue cultures maintained at 33° rather than 37° C.

During the development of the plaque assay for the 229 E virus the effects of temperatures ranging from 30° to 39° C on the development of plaques were investigated. After the normal processes of inoculation and overlaying, monolayers of L 132 cells were maintained at various temperatures by submersion in waterbaths. After 4 and 5 days of incubation, plates were removed, fixed and stained. Table 4 shows that no plaques were formed at 30° or 39° C during this period. The plaque counts at 33° and 35° C were similar, but at 37° C there were less plaques (60% for 229 E and 50% for LP).

Table 4. *The Effects of Various Temperatures Between 30° and 39° C on the Production of Plaques in L 132 Cells by 229 E and LP Viruses*

Temperature (°C)	Plaque Count (average of 3 plates)			
	Virus 229 E		Virus LP	
	Incubation Period		Incubation Period	
	4 days	5 days	4 days	5 days
30	0	0	0	0
33	66	89	26	29
35	49	86	19	24
37	6	33	8	14
39	0	0	0	0

To investigate the effects of temperature on the replication of coronaviruses during a single infectious cycle, cells were infected with either 229 E or LP viruses at 33° C and then divided into aliquots which were maintained at various temperatures between 30° and 39° C. Figures 3 and 4 shows the results, and the eclipse periods and 12 hour yields are given in Table 5.

The effects of this range of temperature on the two viruses was basically the same. The depression in the yield of LP virus at 30° compared with that at 33° C

Table 5. *The Length of the Eclipse Period and the Maximum Yield of 229 E and LP Viruses Grown in L 132 Cells at Various Temperatures (see also Figs. 3 and 4)*

Temperature (°C)	229 E		LP	
	Eclipse Period (hours)	Yield (p.f.u./IC)	Eclipse Period (hours)	Yield (p.f.u./IC)
30	8	1.5×10^{-1}	10	5.5×10^{-2}
33	6	1.6	7	2.9
35	5	1.8	6	2.9
37	6	1.9×10^{-1}	7	1.3×10^{-1}
39	>24	$<1.0 \times 10^{-5}$	>24	$<1.0 \times 10^{-4}$

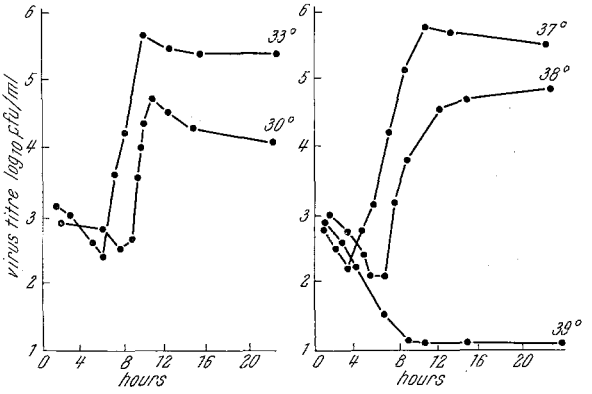

Fig. 3. The growth of 229 E virus in suspended cell cultures at various temperatures, showing the cut-off in virus production between 38° and 39° C (see also Table 5)

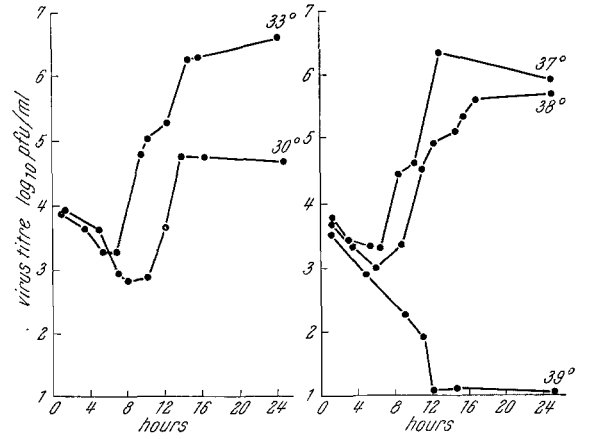

Fig. 4. The growth of LP virus in suspended cultures of L 132 cells, under similar conditions for the 229 E growth curves (see previous Figure; see also Table 5)

was 140-fold; the corresponding depression for 229 E was only 20-fold. The eclipse phases were about 1 hour shorter at 35° than at 33° C; for LP it was lengthened from 7 hours at 33° to 11 hours at 30° C. For 229 E virus the corresponding increase was from 6 hours to 8 hours. There was substantial inhibi-

tion of both viruses at 38° and complete inhibition at 39° C. Thus, there was evidence of both high (39°) and low (30°) temperature suppression of these viruses.

3.4. Attempts to Locate the Timing of the 39°-Sensitive Event

Because of the marked depression in the yields of 229 E and LP viruses at 39° C, it was possible to perform "shift" experiments in which infected cell cultures were moved from a "permissive" (33°) to a "restrictive" (39°) temperature and *vice versa*.

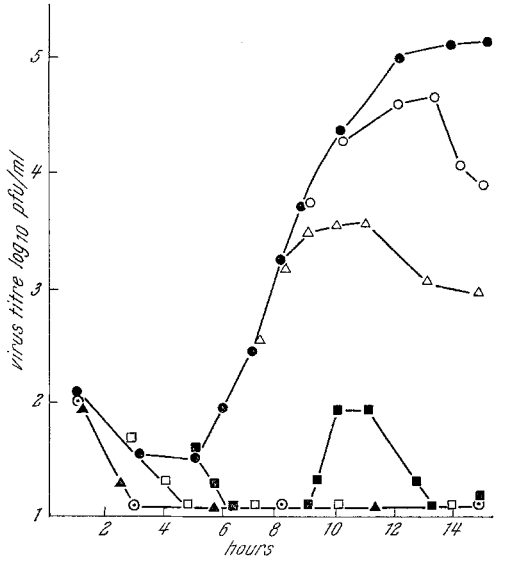

Fig. 5. The effects on the formation of 229 E virus when infected cultures of L 132 cells are removed from 33° to 39° C at various times after infection

● —— ● 33° only, ◉ —— ◉ 39° only

Shifted from 33° to 39° C at: 1 hour ▲ —— ▲, 3 hours □ —— □, 5 hours ■ —— ■, 7 hours △ —— △, 9 hours o —— o

The results of a "shift-up" experiment with 229 E virus are shown in Figure 5. Replicate suspension cultures of infected L 132 cells were shifted from 33° to 39° C at various intervals up to 9 hours after infection. Cell suspensions were sampled throughout the experiment at hourly intervals. Shifting infected cells to 39° C within 5 hours of infection prevented any subsequent formation of infectious virus. The cell suspension shifted at 5 hours produced a small quantity of virus (10^2 p.f.u./ml). The rate of virus production in the cell suspension which was shifted to 39° C at 7 hours was similar to that of the control (33°) suspension for the next two hours, but reached an early plateau. The culture shifted at 9 hours produced only 20% of the control yield of virus.

For "shift-down" experiments, cells were infected at 33° C and immediately transferred to 39° C after subdivision into replicate suspensions. They were then moved back to 33° C at various times up to 9 hours. The duration of the eclipse period increased proportionately with the increasing delay in shifting the cultures back to the permissive temperature (Fig. 6). Also, after a minimum of 1 hour at 39° C, all cultures produced a reduced yield when compared with a control which

had been kept at 33° C only. The reasons for the extra depression in the yield of LP virus in the culture shifted at 5 hours (Fig. 6) are not known.

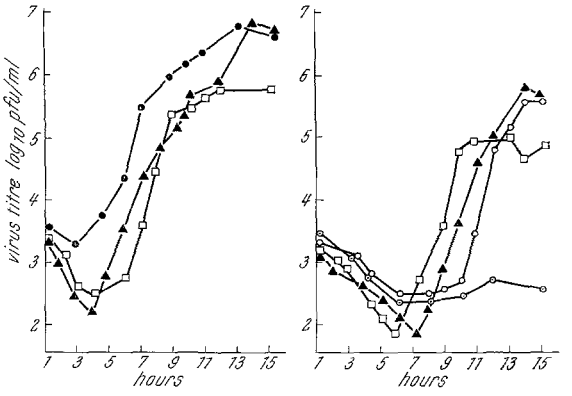

Fig. 6. The results of a "shift-down" experiment with LP virus in which infected L 132 cells were transferred from 39° to 33° C at various times after infection
● —— ● kept at 33°, ◉ —— ◉ kept at 39° C.
Shifted from 39° to 33° C at: 1 hour ▲ —— ▲, 3 hours □ —— □, 5 hours ■ —— ■, 7 hours △ —— △, 9 hours o —— o

4. Discussion

4.1. Adsorption of Virus and Formation of Infectious Centres

In all the experiments reported in these studies, the initial proportion of L 132 cells infected with either of the two coronaviruses studied was low and rarely exceeded 20%. The adsorption of virus at 4° C was very poor; under similar conditions L 132 cells adsorbed rhinoviruses much more efficiently and gave rise to the expected number of infectious centres (Stott and Heath, 1969). Even at 33° C the adsorption of coronaviruses was still relatively inefficient and the numbers of infectious centres formed were below the theoretical levels predicted from the adsorbed multiplicity. This multiplicity could be artificially high if thermal inactivation of virus occurred during the adsorption period. However, virus maintained in cell-free medium for the same period at 33° C showed no detectable loss of infectivity. On the basis of trypan blue staining very few dead cells were detected in cell suspensions before virus was added. Therefore adsorption to dead cells was considered unlikely. Whether or not some portion of the cells were infected but unable to produce infectious virus, was not known.

The experimental evidence indicates that some form of autointerference may be operating under the conditions of adsorption. One characteristic of coronaviruses appears to be their high physical to infectious particle ratio which may exceed 10^4 (Mrs. J. D. Almeida, personal communication). An autoinhibition effect has been observed when undiluted egg fluids containing infectious bronchitis virus are passaged serially (McDougal, personal communication). The large proportion of uninfectious virus particles may be interfering with the adsorption or replication of the remainder. This could involve the direct blocking of cell receptors by inactive virus or some viral component. Purified preparations of coronaviruses seem to contain large numbers of particles with a structure which resembles the

club-shaped projections of the viruses; these might be the receptor-blocking antigens.

4.2. The Viral Growth Cycle

There appears to be a reasonable correlation between the sequence of events determined in these studies and those made on other coronaviruses. Infectious LP and 229 E viruses reappeared in L 132 cells between 4 and 6 hours after infection, at 33° C. BECKER *et al.* (1967) first observed 229 E and AIB particles in WI-38 cells, and in cells of the chorioallantoic membrane of 10-day-old eggs, respectively, after a period of 10—12 hours postinfection.

DAVID-FERREIRA and MANAKER (1965) were able to detect particles of mouse hepatitis virus 7 hours after infection of NCTC 1469 mouse liver cells in suspension culture.

4.3. The Effects of Temperature

The absence of virus replication at 39° C could be due to a high rate of thermal inactivation at this temperature. However, separate experiments have determined that the rate of thermal inactivation at 39° C (in a protein-free medium) was 0.18 log p.f.u./hour, less than twice the rate at 33° C. The yields of viruses from cells maintained at 39° C were at least 500-fold less than at 33° C, and there was also a 100-fold depression in yields at 30° C for both LP and 229 E viruses. It was concluded that the effects of temperature were mainly on virus replication within the cell.

The shift experiments failed to accurately locate any one temperature sensitive event which is subject to inhibition at 39° C. The shifting of cultures from 33° to 39° C earlier than 5 hours after infection completely cuts off virus production, therefore the sensitive reaction would appear to be operational late in the eclipse phase. Shifting of infected cultures after the normal eclipse phase still resulted in a depressed yield which would suggest an inhibition of a continuing process, rather than of a specific stage in virus replication. The shift-down experiments indicate that the viral genome is able to survive in some intracellular form for at least 9 hours but, because of the reduced virus yield, the genome may be subjected to increased degradation by cellular cathepsins (LWOFF, 1969). Some stages in virus replication seem to be able to proceed at 39° C as there is, on the average, only a 1½ hour period after shifting infected L 132 cells from 39° to 33° C, before new infectious virus is formed and the normal eclipse period does not have to elapse before the formation of complete virus.

Because of the non-optimal conditions, such as the low percentage of cells infected initially, the interpretation of these experiments can only be provisional until conditions of synchronious infection and replication of these viruses can be obtained.

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