CHARACTERISTICS OF THE ADENOVIRUSES

III. Reproductive Cycle of Types 1 to 4*

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Investigation of the early period of viral multiplication permits observation of certain discernible stages in the reproductive cycle. Detailed analysis of these steps ((*a*) combination of virus with susceptible cells, (*b*) synthesis of new viral particles, and (*c*) dissociation or release of virus) yields data concerning basic characteristics of the agent. This information is pertinent for purposes of classification, and to stimulate hypotheses concerning the nature of the reactions of a virus with susceptible host cells under conditions of natural infections. To obtain evidence concerning the characteristics of multiplication of the adenoviruses (1–3), investigation of 4 prototype viruses of this group was undertaken. A preliminary report of these studies has been made (4); detailed presentation of the experimental results forms the basis of this paper. These data indicate that the types 1 to 4 adenoviruses studied adsorb at a relatively slow rate to the host cells, strain HeLa cells; that the latent or eclipse period of the multiplication cycle is relatively long and is longer for types 1 and 2 than for types 3 and 4; that the incremental period of viral propagation is relatively short; and that only a very small amount of the maximum virus detectable is dissociated from the host cells during and after the incremental phase of the multiplication cycle.

Materials and Methods

Tissue Culture.—A strain of HeLa cells was employed throughout this investigation (5). For the majority of experiments, a growth fluid composed of 40 per cent human serum and Hanks's balanced salt solution (BSS) was used for propagation of cells in serial cultures by methods described in detail previously (6). In the latter part of this investigation cells were cultivated in Eagle's basal medium (7) to which was added human serum in a final concentration of 10 per cent. These cells, as compared with those previously employed, were equally susceptible to adenovirus infection. Cell cultures were initiated by inoculation of 30 ml of a

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cell suspension containing 50,000 cells per ml. into 32 oz. duraglass bottles. Cultures of cells were also prepared in screw-top tubes with 0.5 ml. of a HeLa cell suspension containing 100,000 cells per ml. Throughout this investigation the fluid employed for preparation of tube cultures consisted of 40 per cent human serum and 60 per cent BSS. Test tube cultures were used after 24 hours of incubation and contained 40 to 70 thousand cells. All cell suspensions were prepared as routine by mechanical disruption of cell sheets and the number of cells in suspension was counted in a standard hemocytometer.

To determine the mean number of cells in the tissue cultures prepared in tubes for use in viral multiplication experiments, 4 uninoculated cultures were selected. Uniform single cell suspensions were prepared by the use of the chelating agent, versene (ethylenediaminetetraacetic acid). Tissue culture fluid was removed from each culture and 0.5 ml. of versene, 0.05 per cent by weight in 0.85 per cent sodium chloride, was added. After 10 to 15 minutes the cell suspension was prepared by pipetting 15 to 20 times with a 1 ml. pipette. Cells were then counted in a hemocytometer. The geometric mean of the 4 cell counts was computed and is expressed as the mean cell count per culture.

Viruses.—Types 1 to 4 adenoviruses were employed. Types 1 to 3 were obtained from Dr. R. J. Huebner, National Institute for Allergy and Infectious Diseases; type 4 (RI-67 agent) was supplied by Dr. M. R. Hilleman, Walter Reed Army Institute of Research. Viruses were propagated in HeLa cell cultures which had been washed 3 times with 50 ml. of BSS to remove human serum. The washed cell sheets were then overlaid with 40 ml. of maintenance mixture composed of 67.5 per cent of Scherer's amino acid–vitamin solution (5), 25 per cent tryptose phosphate broth, and 7.5 per cent chicken serum (8). Cultures were inoculated with $10^{3.0}$ to $10^{4.0}$ 50 per cent infectious doses of virus (ID_{50}) and incubated at 36°C. until the HeLa cells had undergone complete cytopathic changes (3 to 4 days). The infected cells and maintenance mixture were harvested, frozen and thawed 6 times to disrupt the cells, and cell debris removed by centrifugation. Viral suspensions were stored at −70°C. in sealed glass ampules.

Infectivity Titrations.—Titrations were done in test tube cultures of HeLa cells which had been washed 3 times with 2.0 ml. of BSS. To each tube was added 0.8 ml. of a maintenance mixture identical with that employed for viral propagation. Serial 1:3.2 ($10^{-0.5}$)-fold dilutions of viral suspension were prepared in BSS, and into each of 2 tubes 0.1 ml. of each dilution was pipetted. At least 5 dilutions were inoculated in each titration. Cultures were incubated at 36°C. for 6 days. A culture was considered to be infected when at least 20 per cent of the cells had undergone characteristic viral cytopathic changes. When more than one definite virus-produced plaque was observed, but the area of cells involved was less than 20 per cent of the total culture, it was called a plus-minus; when both tubes inoculated with a single dilution were plus-minus, the dilution was scored as having infected 1 out of 2 cultures. The infectivity titer is defined as the highest dilution of virus in a 0.1 ml. inoculum which infected 50 per cent of the tubes (9), and is expressed as one 50 per cent infectious dose (ID_{50}).

EXPERIMENTAL

Reproducibility of Infectivity Titrations with Type 4 Adenovirus.—As a preliminary to the investigation of the quantitative aspects of the multiplication cycle of adenoviruses it was necessary to assess the reliability of techniques available to measure the quantity of infectious virus in infected fluids. Initial studies indicated that end points of infectivity titrations using 2-fold or 1:3.2-fold serial dilution techniques were sharp, so that a change from positive in all tubes receiving a single dilution to negative in all inoculated cultures occurred over only 2 or 3 dilution increments. It then appeared likely that inoculation

of relatively few tubes (2 to 4) with each dilution would yield highly reproducible results. The following experiment was designed to determine the validity of this assumption.

Twelve individual titrations were done with a single pool of type 4 adenovirus. Serial 1:3.2-fold ($10^{-0.5}$) dilutions were prepared and 0.1 ml. of each dilution was inoculated into each of 2 culture tubes. All titrations were done on the same day with tissue culture tubes prepared with a single HeLa cell culture approximately 24 hours before they were employed. The infectivity titers were tabulated and were shown to have a normal distribution. The reproducibility of the end point was determined as described by Lauffer and Miller (10).

The essential data from this experiment and the results of the appropriate computations are presented in Table I. It will be noted that titration end points were highly reproducible; the mean deviation from the geometric mean was

TABLE I

Reproducibility of Infectivity Titers with Type 4 Adenovirus in HeLa Cell Cultures

No. of infectivity titrations	Infectivity titer type 4 virus			Deviation from geometric mean			Standard deviation σ
	Lowest	Highest	Geometric mean	Least	Greatest	Mean	
	log	*log*	*log*	*log*	*log*	*log*	
12	−3.75	−4.25	−4.02	−0.02	−0.27	±0.075	0.129

± 0.075 and the standard deviation was 0.129, or 1.35-fold. From these computations it can be shown that with any 2 individual end points a difference of 0.36 log unit should occur by chance only once in 20 times (10). Or expressed somewhat differently, if 2 viral suspensions have infectivity titers which differ by greater than 0.36 log unit, the probability that the quantity of infectious virus in the 2 suspensions is similar is about 0.05. The results of this experiment indicate that using 1:3.2 ($10^{-0.5}$) dilution increments, one can inoculate as few as 2 cultures with each dilution and obtain highly reproducible and reliable end points.

Adsorption of Adenoviruses to HeLa Cell.—To react with a host cell, association of virus with cell must first occur. The events subsequent to the initial reaction are dependent upon the number of viral particles which successfully combine with the susceptible cells and the speed with which the reaction is accomplished. Two different types of experiments were devised to investigate this initial phase of the multiplication cycle.

A. Direct Measurment of Viral Adsorption.—

A solid sheet of HeLa cells was grown on the flat side of a 32 oz. duraglass bottle. After removal of growth fluid, the cells were washed 3 times with BSS containing 2 per cent chicken serum. The cell sheet was overlaid with 15 ml. of virus diluted in maintenance mixture to contain approximately $10^{2.0}$ to $10^{3.0}$ ID_{50} per 0.1 ml., and the cultures incubated at 36°C.

An aliquot of the diluted viral suspension was frozen immediately and stored at −30°C.; a second aliquot of virus was incubated at 36°C. for the experimental period and then stored at −30°C. After specified periods, 1.0 ml. aliquots of the viral suspension were removed from the culture, frozen, and stored at −30°C. Every 30 to 45 minutes during the adsorption period the cell-virus mixture was gently agitated. To determine the quantity of virus which combined with HeLa cells, *2-fold* dilutions of the supernatant fluids were made, and infectivity titrations were done. With each experiment 2 to 4 similar cultures of HeLa cells were employed to estimate the number of cells in the adsorption flask.

The results of experiments carried out with types 1 to 4 adenoviruses, summarized in Table II, indicate that combination of virus with HeLa cells

TABLE II

Adsorption of Adenoviruses (Types 1 to 4) to HeLa Cells

Adsorption period	Type 1		Type 2		Type 3		Type 4	
	Titer	Per cent adsorbed	Titer	Per cent adsorbed	Titer	Per cent adsorbed	Titer	Per cent adsorbed
hrs.								
0*	4096	—	4096	—	64	—	544	—
1	2048	50.0	1536	63	64	0	512	0
2	2048	50.0	2048	50	96	0	512	0
3	2048	50.0	1536	63	32	50.0	256	53
4	2048	50.0	2048	50	32	50.0	256	53
5	1024	75.0	2048	50	16	75.0	192	65
6	1536	67.5	1024	75	24	67.5	128	77
7	1536	67.5	1024	75	16	75.0	256	53
8	1536	67.5	768	82	8	87.5	192	65
11 to 12	2048	50.0	2048	50	16	75.0	256	53

* Geometric mean of control heated at 36°C. for experimental period and control frozen at commencement of experiment.

was accomplished slowly. By the techniques employed, initial adsorption of virus to HeLa cells could be detected first with types 1 and 2 viruses. Maximum viral adsorption required 5 to 6 hours, and no significant differences were noted in any of the 4 viruses tested. In an occasional experiment with types 1 and 3 viruses, maximum adsorption was noted in 4 hours. The differences observed in viral types were not consistent, and probably resulted from cell variations or technical difficulties which were not recognized. Moreover, the quantity of measurable virus adsorbed was small relative to the number of cells available. It may be computed that only one infectious unit of virus was adsorbed per 200 to 1000 HeLa cells. These ratios were not altered significantly when larger quantities of virus (undiluted infected tissue culture fluid) were used. Furthermore, the percentage of virus adsorbed was not increased when the number of cells was augmented or the quantity of virus inoculated decreased.

The use of cell suspensions rather than cell sheets also did not alter the percentage of total quantity of virus adsorbed. In addition, neither the rate of viral adsorption nor quantity of virus combined with host cells was increased when adsorption was done at 4°C. or room temperature, or when the menstrum utilized was distilled water, 0.85 per cent NaCl, 0.85 per cent NaCl buffered at pH 7.2 with 0.01 M phosphate or 0.05 per cent versene. These fluids usually caused separation of many cells from the glass surface, but viral adsorption was not altered. When the reaction was carried out with tube cultures, rotation of the tubes at a rate of 12 times per hour did not augment viral adsorption.

B. Indirect Measurement of Viral Adsorption.—

The above data suggested that the techniques employed might be insensitive and unsuited to detect adsorption of relatively small quantities of virus from the supernatant fluid. To determine the validity of this possibility, experiments were done in which viral adsorption was measured by indirect means; *i.e.*, the rate of development of cytopathic effects in cells exposed to virus for varying periods.

For every virus tested each of a number of HeLa cell tube cultures was inoculated with 0.1 ml. of undiluted viral infected tissue culture fluid containing $10^{2.0}$ to $10^{3.5}$ infectious doses. Immediately after inoculation the fluids from 4 tubes were removed, each tube was washed 3 times with 2.0 ml. of BSS containing 5 per cent chicken serum, and 1.0 ml. of maintenance mixture was added. The remainder of the cultures was incubated at 36°C., and at specified times the infected fluids from 4 tubes of each series were removed and the tubes similarly handled. All cultures were examined microscopically daily and the per cent of cells which had undergone characteristic viral cytopathic changes was estimated. On the day of the initiation of the experiment an infectivity titration of each viral inoculum was done. The day on which approximately 50 per cent of the cells in each culture were cytopathic was estimated, and the mean time for the 4 cultures in each group computed.

The results of 2 experiments, summarized in Fig. 1, indicate that some of each of the 4 types of adenoviruses tested was combined with HeLa cells in as short a period as could be determined. From data which relate rate of viral multiplication to rate of development of cytopathic alterations (presented below) it seems reasonable to assume that in these experiments the time required for 50 per cent of the cells to show characteristic pathological changes can be correlated with the quantity of virus adsorbed by the cells. Therefore, although some virus was adsorbed almost immediately, the quantity was probably not great, and the change in titer of the supernatant fluid was too small to be detected by the dilution technique for infectivity titrations. It might be implied from these data that types 1 and 3 viruses were adsorbed faster than types 2 and 4, but the variations in quantity of each virus inoculated probably account for these differences. Since the data obtained are in effect estimates, it would appear that viral adsorption commenced immediately after viral inoculation and that maximum adsorption required at least 2 hours and probably

4 to 6 hours. When the 2 methods employed to investigate combination of adenoviruses with HeLa cells are compared, it seems probable that the early period of adsorption, when the largest quantity of virus was in the fluid phase of the reaction, was most accurately studied by the indirect technique, and that

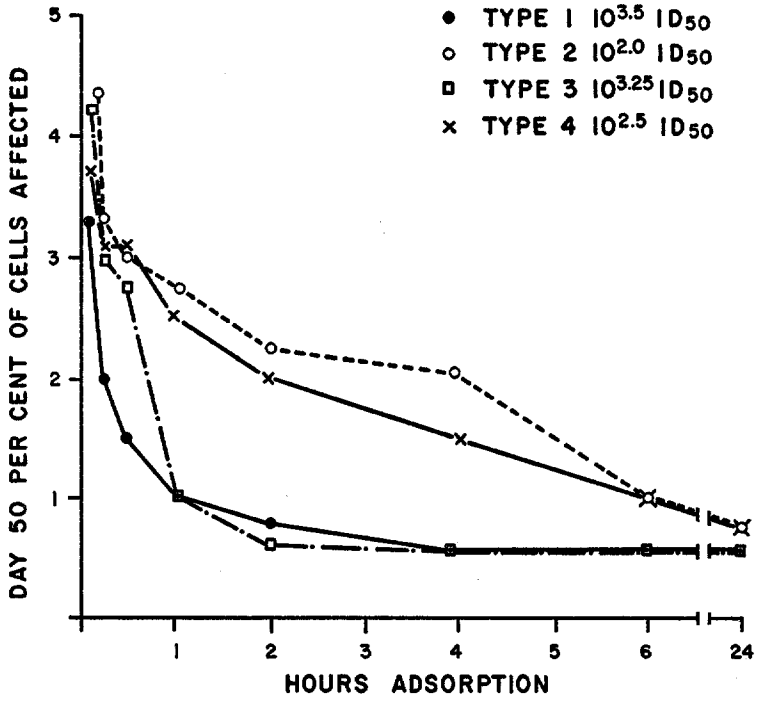

FIG. 1. Rate of adsorption of types 1 to 4 adenoviruses as measured by rate of development of cytopathic effect on HeLa cells. At the indicated times after viral inoculation (adsorption period), culture fluids from 4 tubes were removed, cells washed 3 times, and maintenance mixture added. The day on which approximately 50 per cent of the cells in each culture were cytopathic was estimated and considered the endpoint. The points presented are the means from 4 cultures.

the later period of the adsorptive process was best measured by direct determination of the amount of virus remaining in the infected culture fluid.

Multiplication of Adenoviruses

Association of virus with a susceptible host cell is thought to be followed by viral penetration into the host environment. Subsequent to these processes a period of synethsis of new viral material, *i. e.* viral propagation, ensues. It has been demonstrated with a number of animal and bacterial viruses that viral multiplication is a cyclic process occurring individually in each cell. As virus spreads from cell to cell the cycles become asynchronous and a continuous

production of new virus is observed. Experiments with adenoviruses were done to describe the characteristics of the initial cycle of multiplication as well as to determine the rates of viral multiplication when decreasing quantities of each virus were employed.

Initial Cycle of Multiplication.—The characteristics of the multiplication of a virus can probably be measured best by an investigation of the latent and incremental periods of the initial cycle of multiplication. For such studies it is necessary to infect simultaneously as many cells as possible. Because of the relatively slow rate of adenovirus adsorption and the marked toxic effect on HeLa cells, however, it was not possible to accomplish this ideal circumstance, but rather only approximately 0.1 of the host cells were infected during the adsorption period.

A large number of HeLa cell tube cultures were inoculated with 0.1 ml. of 10^{0} to $10^{-2.0}$ dilution of infected tissue culture fluid containing $10^{4.0}$ to $10^{4.6}$ infectious doses of virus. A $10^{-1.6}$ or $10^{-2.0}$ dilution of types 1 and 2 viruses was employed to reduce toxic effects of these agents. After incubation at 36°C. for 4 to 6 hours the infected fluid was removed, the cells were washed with 5 ml. of BSS containing 3 per cent chicken serum to remove the majority of the unadsorbed virus, and 1.0 ml. of maintenance mixture was added. More extensive washing of the cells was not done, because disruption of the cell sheets frequently resulted from multiple washings after infection with large quantities of virus (particularly with types 1 and 2). At specified times after viral inoculation (from 4 to 48 hours), the cells and fluid from 4 tubes were pooled, frozen, and stored at −30°C. until the completion of the experimental period. Infectivity titrations as previously described were done on all supernatant fluids from cell suspensions frozen and thawed 6 times. Infectivity titration of the viral inoculum was carried out at the commencement of each multiplication cycle experiment. The number of cells in 4 uninfected cultures was determined individually after the second series of washes (6 hours after the onset of the experiment), and the geometric mean of the 4 counts was computed.

The results imply that adenoviruses, like other animal viruses (11–16), have a cyclic form of reproduction. The data from experiments with all 4 viruses, summarized in Fig. 2, indicate that the latent or eclipse period of the multiplication cycle was relatively long, whereas the incremental period of viral propagation was comparatively short. It is striking that the latent periods for types 1 and 2 viruses were approximately 17 hours, whereas for the related types 3 and 4 viruses the latent periods were significantly shorter, lasting about 14 to 15 hours. These data were obtained in 3 experiments for types 1 and 4 viruses and 2 experiments for the types 2 and 3 agents. Although the period before synthesis of new virus could be detected was longer for type 1 or 2 than type 3 or 4 virus, the rate of viral multiplication for types 1, 2, and 4 viruses was similar. The incremental period for types 1, 2, and 4 viruses was also approximately the same for each agent: 5 to 6 hours. For the type 3 virus, however, the logarithmic period of propagation was somewhat longer, 9 to 10 hours, and the rate of viral multiplication during the incremental period appeared slower.

The experiments described above were done with HeLa cells propagated in a

mixture of Eagle's basal medium and 10 per cent human serum (7). The original studies of the initial cycle of multiplication of types 1 to 4 adenoviruses were done with cells cultured in a growth medium composed of 40 per cent human serum and 60 per cent Hanks's BSS (17). Although the essential characteristics

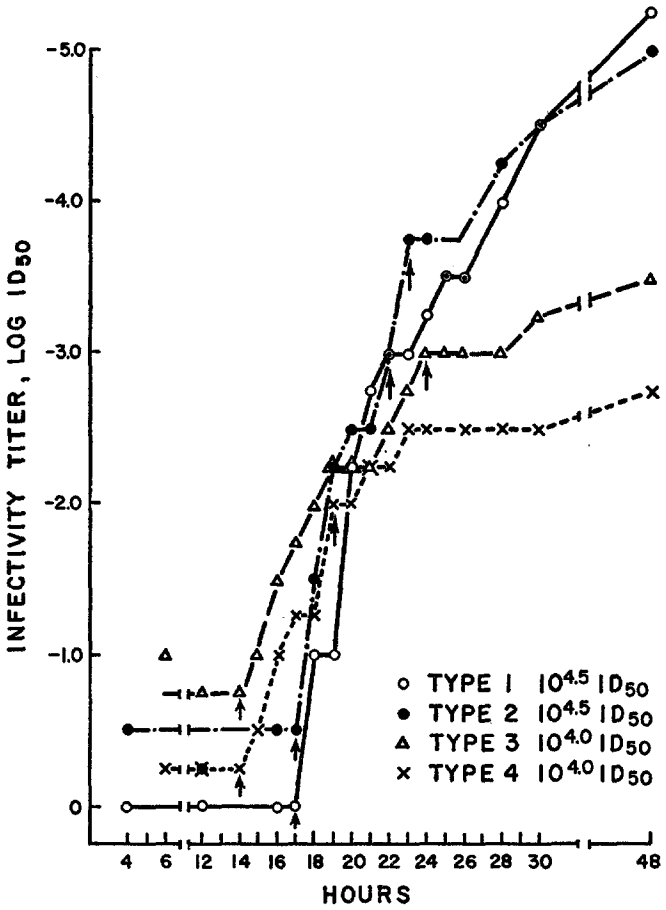

FIG. 2. The initial cycle of multiplication of types 1 to 4 adenoviruses. Four to 6 hours after viral infection, culture fluids were removed, cells were washed 1 time, and maintenance mixture added. At indicated times after viral inoculation cells and fluids from 4 tubes were pooled and employed for infectivity titrations. The arrows on each growth curve indicate the limits of the estimated incremental period.

of the multiplication cycles of types 1 to 4 adenoviruses described above were similar to those previously reported (4), the latent or eclipse periods of the cycles graphically summarized in Fig. 2 were significantly shorter than those described in the prior communication (4). The relative difference between the

latent periods for types 1 and 2 viruses on the one hand and types 3 and 4 on the other is similar with either cell strain although the absolute time period is significantly different for each virus. The shorter latent periods noted in the most recent experiments are considered to be due to an alteration in the HeLa cells as a result of continued propagation in Eagle's basal medium and human serum, although definitive proof of this postulate is not possible because the cells previously employed are no longer available.

Growth Curves with Varying Inocula.—To obtain further evidence concerning the comparative rates of multiplication of the adenoviruses, experiments were done in which viral inocula decreasing in 10-fold decrements were employed, and the development of virus over a period of days was measured. In addition, these studies afforded an opportunity to obtain a rough correlation between the rate of viral multiplication and rate of production of the characteristic cytopathic changes.

Each of the agents under study was diluted in 10-fold decrements and each dilution was inoculated into 36 to 48 HeLa cell tube cultures. For each virus 3 or 4 dilutions were employed. After incubation at 36°C. for 6 hours to permit maximum viral adsorption, the infected culture fluid was removed and each culture washed with 4 ml. of BSS containing 2 per cent chicken serum. To each culture 1.0 ml. fresh maintenance mixture was added. Immediately after the cultures were washed, the cells and fluids from 4 tubes of each dilution series were harvested, pooled, and stored at −30°C. The remaining infected cultures were incubated at 36°C.; at specified times 4 cultures from each series were pooled and stored frozen at −30°C. At the completion of the period of incubation, infectivity titrations were done on supernates from frozen and thawed cell suspensions. During the period of viral multiplication infected cultures were examined daily and the per cent of cells which had undergone cytopathic alterations was estimated. For each agent an infectivity titration of the seed virus was done on the day the multiplication studies were initiated. To compute yield of virus per cell, the number of cells in each of 4 uninfected cultures was enumerated after the second series of washes, and the geometric mean of the 4 cultures was determined.

The data plotted in Figs. 3 to 6 represent the multiplication curves obtained with varying inocula of the 4 types adenoviruses employed. For each virus there is a family of parallel curves, which indicates that with these agents, as with other viruses similarly studied (18, 19), the rate of viral multiplication was independent of the size of the infecting inoculum. Apparent exceptions were occasionally noted (Figs. 5 and 6) when a small amount of virus, $10^{0.5}$ ID_{50} of type 3 or $10^{0.75}$ ID_{50} of type 4 virus, was employed for infection. It was again observed that the viral multiplication rates of types 1, 2, and 4 agents were similar, whereas type 3 adenovirus propagated at a slower rate.

The comparison of the rate of development of cellular changes detectable in the gross with the rate of viral multiplication, as summarized in the lower portions of Figs. 3 to 6, indicates that grossly detectable cytopathic alterations, characteristic of adenovirus-infected HeLa cells, were produced at a rate slower than the rate of viral propagation. These results are consistent with those

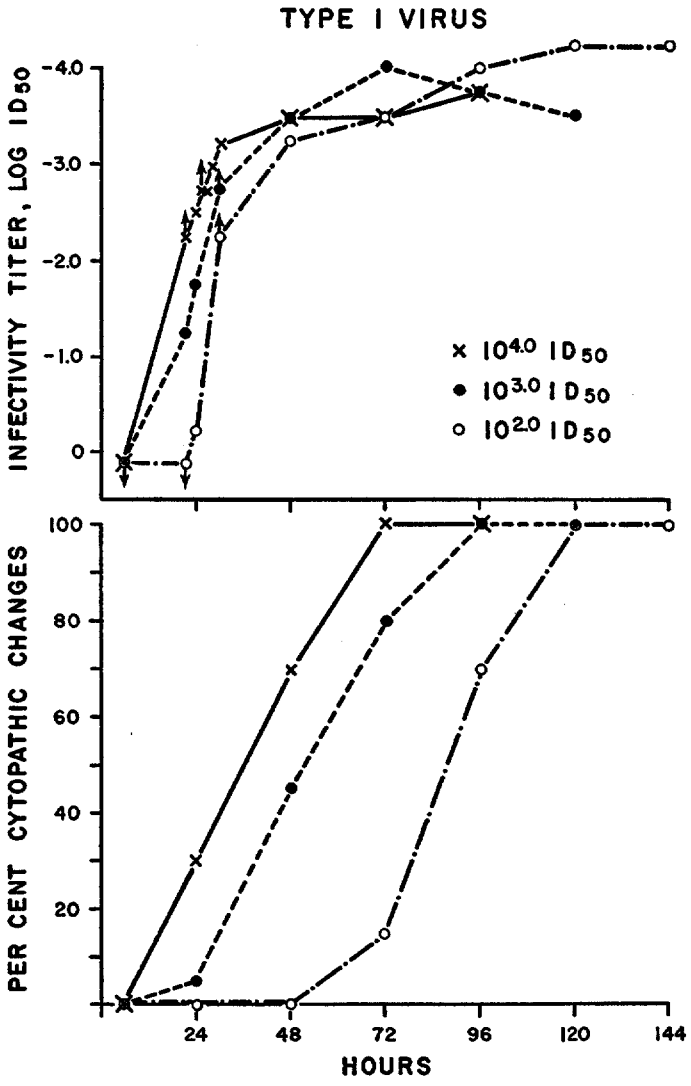

FIG. 3. Multiplication of and cytopathic changes induced by type 1 adenovirus using a viral inoculum containing 10-fold less virus for each series of cultures. Six hours after viral infection, culture fluids were removed, cells washed 2 times, and maintenance mixture added. Each endpoint represents the infectivity titer of the pooled cells and culture fluids from 4 tubes (upper section) and the mean of the estimated per cent of cytopathic cells in 4 tubes (lower section). All viral inocula were used in the same experiment.

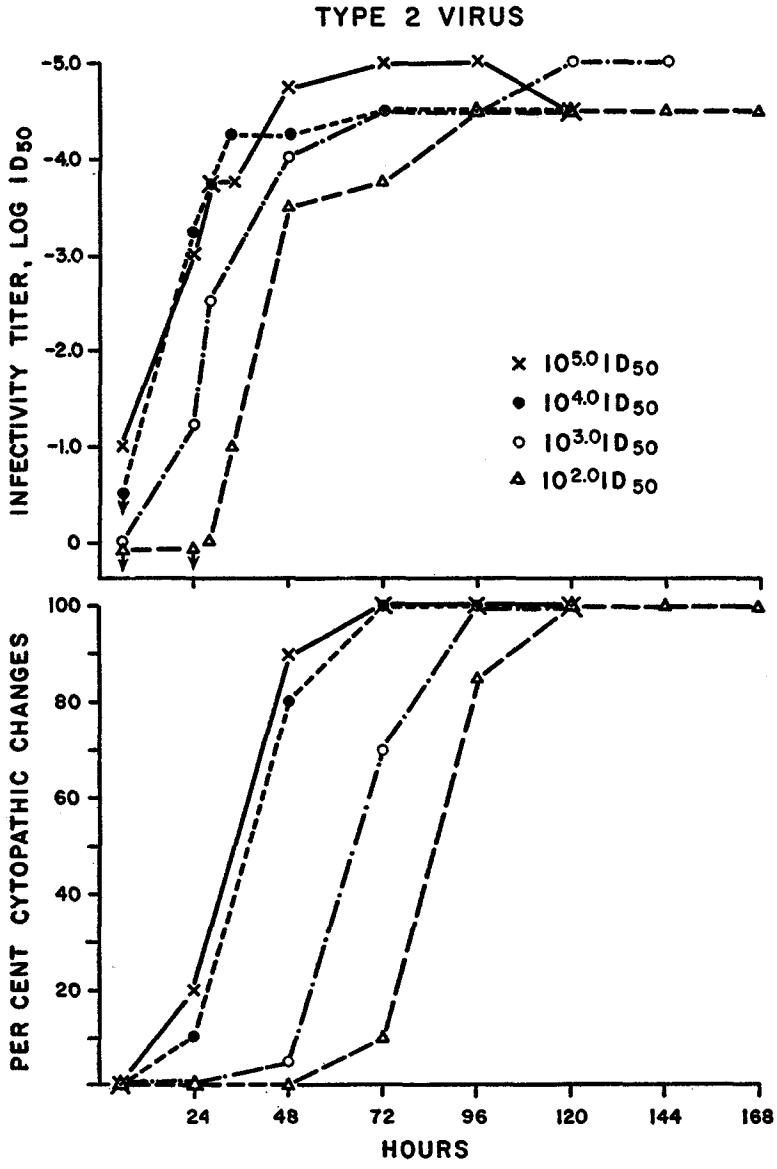

FIG. 4. Multiplication curves and rate of cytopathic changes in HeLa cultures infected with varying quantities of type 2 adenovirus. Experiments were done exactly as described in legend for Fig. 3.

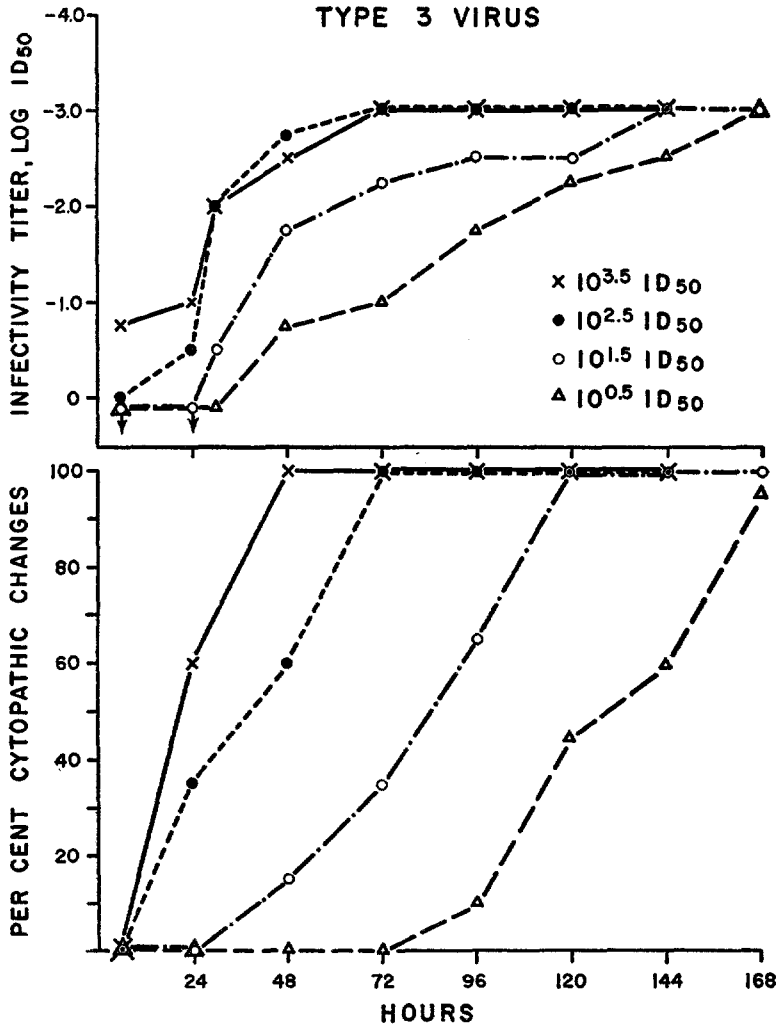

FIG. 5. Multiplication of and cytopathic changes induced by type 3 adenovirus. Each curve represents an inoculum containing 0.1 the quantity of virus used in the preceding series of cultures. Experiments were done exactly as those summarized in Fig. 3.

reported for other viruses (18, 19). The slower rate of development of cytopathic alterations may, however, be only apparent and may be the result of the insensitivity of the method of observation. When fixed and stained preparations of adenovirus-infected HeLa cells were studied, the earliest cellular changes characteristic of viral infection were observed approximately 14 hours after viral inoculation, even before an increase of infectious virus was detected (20).

Yield of Adenovirus.—The studies of the propagation of adenoviruses in

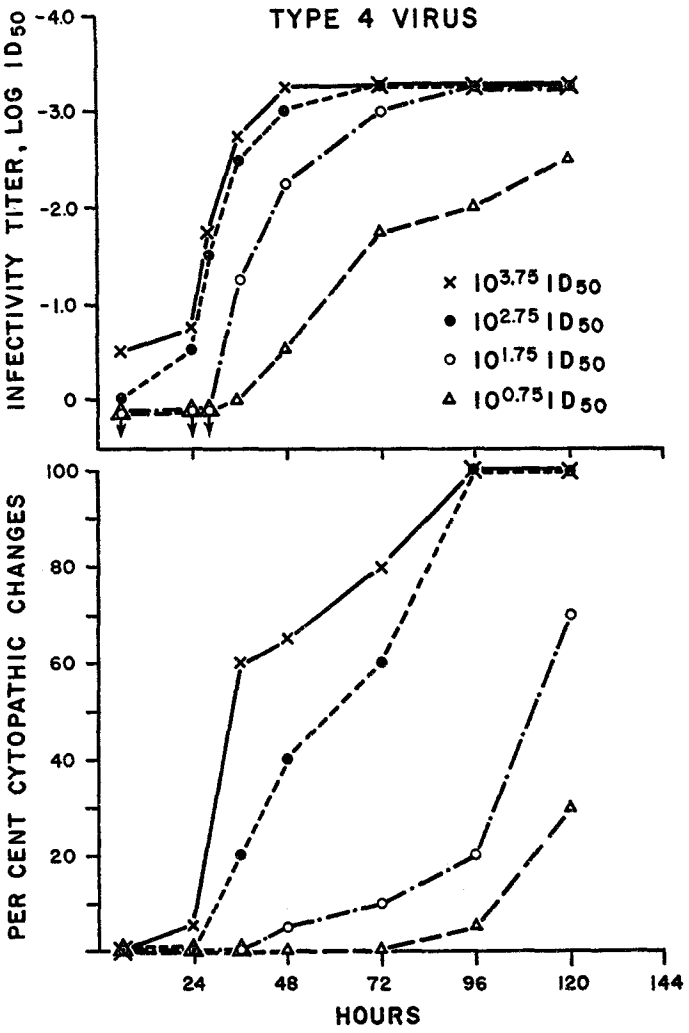

FIG. 6. Multiplication of and cytopathic changes induced by type 4 adenovirus. Each curve represents an inoculum containing 0.1 the quantity of virus employed in the preceding series of cultures. Experiments were done exactly as those described in Fig. 3.

HeLa cell cultures furnished data on the maximum yield of virus and the total quantity of virus produced during any period of the multiplication cycle. From these data and the information on the number of cells present in each culture, the yield of virus per host cell was readily computed. The results, summarized in Table III, were obtained from the data presented in Figs. 2 to 6. These findings express the relatively small amount of type 3 or 4 virus produced by

each cell; the low virus yield is indicated in another manner by the low infectivity titers of completely cytopathic cultures. Although the yield of virus per cell may vary from culture to culture, so that the figures presented in Table III should not be considered as constant values, the variations observed have not been greater than ±2 to 3 times the figure presented. The greater amount of types 1 and 2 viruses synthesized in each cell as compared to the types 3 and 4 agents was a consistent finding with the strains employed, and was reflected also in the greater infectivity titers of types 1 and 2 adenoviruses suspensions.

TABLE III

Yield of Adenovirus, Types 1 to 4, per HeLa Cell

Virus	No. cells per ml.	Virus yield, ID_{50} per ml.	Virus yield, ID_{50} per cell
		log	
Type 1*	4.8×10^4	5.25	3.7
Type 1‡	6.1×10^4	6.25	29.2
Type 2§	5.3×10^4	6.00	18.9
Type 2‡	5.9×10^4	6.00	17.0
Type 3‖	4.8×10^4	4.00	0.2
Type 3‡	5.2×10^4	4.50	0.7
Type 4¶	4.3×10^4	4.25	0.4
Type 4‡	4.9×10^4	3.75	0.1

* From data presented in Fig. 3.
‡ " " " " " 2.
§ " " " " " 4.
‖ " " " " " 5.
¶ " " " " " 6.

The explanation for the relatively small yield of types 3 and 4 virus from infected HeLa cells is only conjecture at present; possible hypotheses will be considered in the discussion of this paper.[1]

Dissociation of Adenovirus from Infected HeLa Cells.—The early studies of the adenoviruses indicated that a greater viral yield was obtained from an infected culture when the cells were disrupted rather than when the culture fluid alone was harvested (2, 4). Investigation of this phenomenon indicated that the quantity of virus obtained was identical whether the infected cells were disrupted by grinding with alundum in a mechanically driven teflon grinder, by homogenizing in a Sorval highspeed omnimixer, or by freezing

[1] Since the completion of this study a plaque assay technique has been developed to measure quantity of infectious adenovirus. Preliminary studies indicate that the total number of infectious particles in a viral suspension is approximately 10 times that which can be determined by the serial dilution technique employed in the present investigation. It might be considered therefore that the yield of virus per cell was actually about 10 times greater than that presented in Table III.

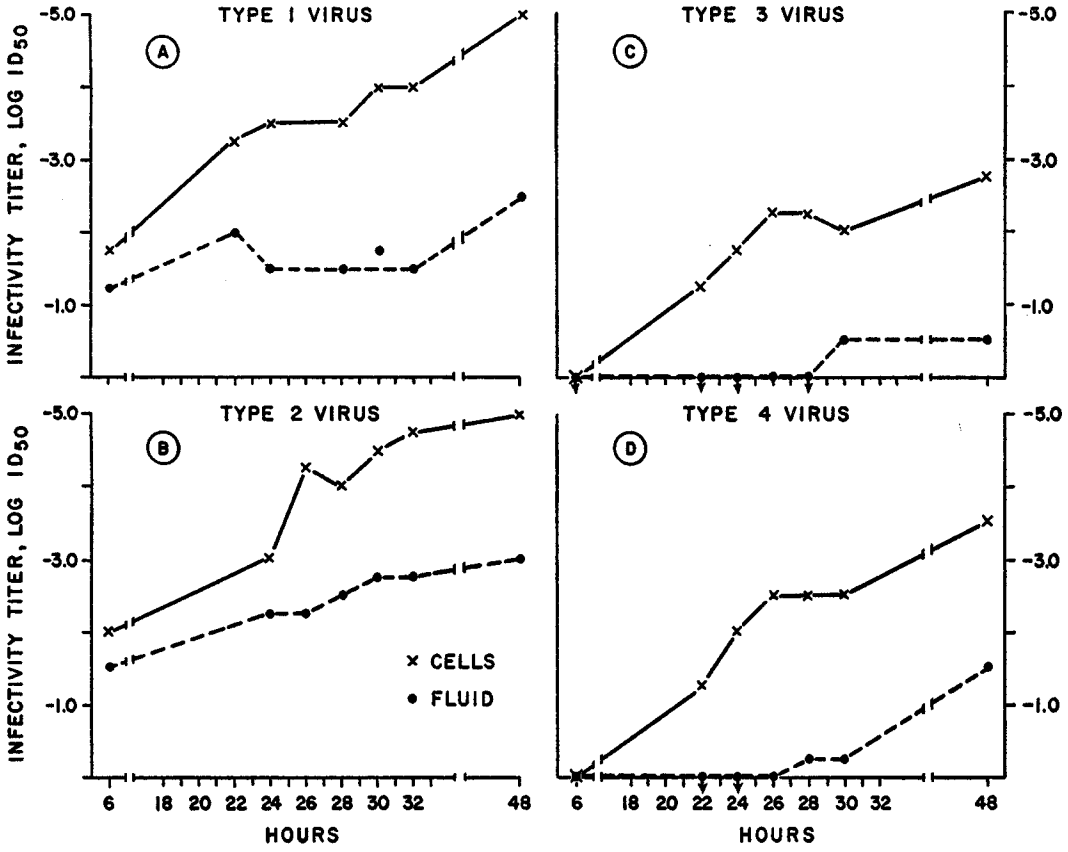

FIG. 7. Infectivity titer of type 1 to 4 adenoviruses in HeLa cells or in culture fluid at varying times after infection. Six hours after viral infection fluid was removed, cells washed 1 time and 1.0 ml. maintenance fluid added to each culture. At indicated times after viral infection fluids from 4 cultures were pooled and centrifuged to remove cells which had fallen from glass. To each culture 1.0 ml. fresh maintenance mixture was added, and cell suspensions from the 4 cultures were pooled. Each endpoint represents the geometric mean of the results of 2 experiments.

7 A, infected with $10^{5.0}$ ID_{50} type 1 virus
7 B, " " $10^{5.6}$ " type 2 "
7 C, " " $10^{3.25}$ " type 3 "
7 D, " " $10^{4.0}$ " type 4 "

and thawing a minimum of 5 or 6 times. To determine whether virus dissociated spontaneously from infected cells, and if so, when dissociation occurred during the multiplication cycle, and the rate of viral release, the following experiments were done.

Twenty-eight to 32 HeLa cell tube cultures were infected with 0.1 ml. of undiluted viral suspension. Cultures were incubated at 36°C. for 6 hours after which the infected fluid was removed and the cells washed once with 4 ml. of BSS containing 2 per cent chicken serum. One ml. of maintenance mixture was added to each tube and all cultures were incubated at 36°C. At specified times the infected maintenance mixture was removed from 4 tubes, pooled,

TABLE IV

Dissociation of Adenoviruses from HeLa cells after Prolonged Infection

Virus type	Infectivity titer*		Comparison of quantity of virus in homogenate and infected fluid	
	Homogenate‡	Fluid§	Ratio H/F‖	Ratio F/H‖
	log	*log*	*fold*	*per cent*
1	−4.00	−2.25	56	1.8
2	−3.50	−2.00	30	3.1
3	−3.25	−1.75	30	3.1
4	−4.00	−2.75	18	5.6

* 6 days after infection with $10^{1.5}$ to $10^{2.5}$ infectious doses of virus. All detectable cells had undergone cytopathic changes.

‡ Infected cells frozen and thawed 6 times; debris removed by centrifugation.

§ Infected culture fluid centrifuged at 2500 R.P.M. for 10 minutes.

‖ H, titer of virus in homogenate.
F, titer of virus in infected fluid.

and centrifuged at 1200 R.P.M. for 15 minutes to remove cells which had fallen from the glass; 1.0 ml. of fresh maintenance mixture was added to each culture, the cells scraped from the glass, and the 4 cell suspensions pooled. All samples were stored at −30°C. until the completion of the growth phase of the experiment. Infected cell suspensions were frozen and thawed 6 times to disrupt the cells and the cellular debris removed by centrifugation. The infectivity titers of all aliquots (wash fluids, supernatant culture fluids, and fluids from frozen and thawed cell suspensions) were determined. The titer of the viral inoculum was measured at the commencement of the experiment.

The results of 2 experiments with each virus are summarized in Fig. 7. At 6 hours after viral infection (and after the infected cells were washed) the quantity of virus in the culture fluid was the result of incomplete removal of unadsorbed virus. During the incremental period of viral multiplication there was practically no increase in viral content of the fluid phase as compared to the rapid rise in quantity of virus in the cells. Indeed, 1.0 per cent or less of the maximum detectable virus was in the fluid phase of the infected cultures even at 48 hours after viral infection with from $10^{3.25}$ ID_{50} (type 3 virus) to $10^{5.0}$ ID_{50} (type 1 or 2 virus), when 90 to 100 per cent of all cells had undergone cytopathic

changes. Moreover, when HeLa cell cultures were infected with $10^{1.5}$ to $10^{2.0}$ ID_{50} of each of the 4 viruses and incubated for 6 days, 6 per cent or less of the greatest amount of virus which could be measured was in the fluid portion of the culture (Table IV). Under these conditions all cells were characteristically rounded and clumped and many had fallen from the glass and become disrupted.

These data clearly indicate that with all 4 types of viruses studied, egress or separation of virus from infected cells did not occur readily, and it is problematical whether spontaneous dissociation of virus from intact infected cells ensued at all. The per cent of virus found in the fluid of infected cultures was always small relative to the quantity obtained by disruption of infected cells, and was dependent perhaps upon the maintenance of cell integrity during the experimental period. The release of some virus into the culture fluid may have been the result of cultural conditions or separation of infected and clumped cells from the glass with subsequent cell lysis, rather than natural release of virus from infected cells.

DISCUSSION

The results of this study indicate that adenoviruses adsorb relatively slowly to HeLa cells, and that the rate of adsorption is not greatly influenced by virus: cell ratio, temperature of the reaction, or electrolyte content of the fluid phase of the reaction mixture. The per cent of the total virus in the reaction mixture which is adsorbed to the host cells is relatively constant, 75 to 87.5 per cent, under the experimental conditions employed. It is not clear, however, whether the very low ratio of infectious units of virus adsorbed per cell (0.001 to 0.005) is a reflection of the relatively small number of infectious units of virus present in the original reaction mixture, the number of viral particles which comprise 1 infectious unit, or the innate characteristics of the viruses.

A unique feature of the adenoviruses studied is the inability of virus to dissociate from host cells to any great extent, even when cells have undergone marked cytopathic alterations. Indeed, it cannot be certain that the small per cent of virus which is released into the fluid phase of the culture does not come from a few infected cells which have fallen from the glass wall of the culture vessel and lysed because of the unfavorable environment rather than from the infected cells which are adherent to the glass. Because of the retention of virus within or on infected cells, and the necessity of disrupting these cells to obtain newly synthesized virus, it is not possible to determine whether the virus measured after cell disruption is the total viral content or only a small proportion of the intracellular virus. Electron microscopic pictures of HeLa cells infected with adenoviruses reveal an exceedingly large number of intranuclear viral-like particles in each thin section (21–23). The vast majority of these aggregated, spherical particles may be non-infectious. On the other hand, if one assumes that all of these particles are infectious, then the relatively low yield of virus

per cultured cell may result from (*a*) failure to release virus from cell material upon mechanical or physical disruption of cells, (*b*) marked aggregation of viral particles when dissociated from the cells, (*c*) inactivation of intracellular virus during the period of viral propagation or (*d*) inactivation by cellular inhibitors upon disruption of the host cells. That the low yield of virus from infected cultures is not due to thermal inactivation during the incubation period is evidenced by the marked stability of the adenoviruses at 37°C. for at least 7 days (24). If the relatively small viral yield per cell (as related to numbers of viral like particles observed in electron micrographs (22–24), is the result of aggregation of the particles, the larger crystal-like aggregates of types 3 and 4 viral particles may serve as at least a partial explanation for the lower yield obtained with these types.

Combined with the characteristics of slow adsorption to and ineffective release from host cells, and perhaps in part a reflection of them, the 4 prototype adenoviruses studied have a relatively long multiplication cycle, 23 to 26 hours. For the strains studied, the similarity of the characteristics of the reactions of the virus with the host cell lends support to the proposed classification of these agents as a single group on the basis of a common complement-fixing antigen, similar host range, comparable cytopathic effects in tissue culture, and like properties of stability on exposure to ether (25, 3).

The data available from this and other studies imply, however, that significant differences do exist between certain of the types of adenoviruses studied: (*a*) the latent period of the multiplication cycle of types 1 and 2 is distinctly longer than that of types 3 and 4; (*b*) the quantitative relationship of types 1 and 2 with their respective homologous type-specific antibody is characteristically different from that of types 3 and 4 (26); and (*c*) the nuclear alterations of HeLa cells induced by types 1 and 2 are markedly different from those caused by types 3 and 4 viruses (20). These studies suggest that there may exist subgroups within the family of adenoviruses. Extension of these studies to other strains of types 1 to 4 adenoviruses, as well as to other types, is necessary to establish the validity of this hypothesis.

SUMMARY

The strains of types 1 to 4 adenoviruses studied attained maximum combination with host cells, strain HeLa, in 5 to 6 hours. During this adsorption period approximately 75 per cent of the total virus became associated with the host cells. Following adsorption of virus to HeLa cells, these agents underwent cyclic multiplication periods similar to bacterial and other animal viruses. The latent or eclipse period of the multiplication cycle for types 1 and 2 viruses was 17 hours, whereas the types 3 and 4 agents had a latent period 14 to 15 hours in length. The rate of viral propagation during the incremental period was very similar for types 1, 2, and 4 viruses, but was slower for the type 3 agent. During

the incremental period of viral propagation newly synthesized virus was not released into the fluid phase of the culture and even after 6 days of viral multiplication when marked cytopathic alterations of the infected HeLa cells had occurred, the spontaneous release was only 2 to 6 per cent of the total virus detectable.

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