INTRACELLULAR LOCALIZATION OF TYPE 4 ADENOVIRUS

I. Cellular Fractionation Studies*

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Electron microscopic studies by several investigators (1–3) have shown virus-like particles in the nuclei of HeLa cells infected with adenoviruses. Morgan and his associates (3) showed disruption of the nuclear membrane in a few cells with release of virus-like particles into the cytoplasm, but did not note viral development in the cytoplasm. A tremendous number of particles have been observed by electron microscopic examination of thin sections of infected HeLa cells, a finding which seems to be out of proportion to the relatively low yield of infectious virus (2) thus suggesting that most of the particles seen are not infectious, or that the large number of intranuclear particles remain aggregated and therefore act as relatively few infectious units. Characteristic nuclear changes in HeLa cells infected with adenoviruses have also been described using light microscopy (4); these alterations have been correlated with formation of specific viral antigen and infectious virus (5). Cytoplasmic changes were also described, but were much less marked and not characteristic (4). Because microscopic observations cannot disclose the site of development of infectious virus, and because it seemed possible that a large proportion of the particles visualized were not infectious, studies were undertaken to determine the intracellular localization of infectious adenovirus. In the experiments described in this report, nuclear and cytoplasmic fractions of HeLa cells infected with adenoviruses were obtained by mechanical disruption and differential centrifugation, and infectious virus in these fractions was measured.

The results obtained indicated that the majority of infectious type 4 adenovirus was isolated from the cytoplasm of the HeLa cell; experiments done with type 1 adenovirus, although not conclusive, yielded somewhat different results. The techniques used in these experiments have many limitations and

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the data obtained were carefully considered with reference to these limiting factors.

Materials and Methods

Tissue Culture.—Epithelial cells of the HeLa strain (6) were employed in all experiments and were propagated in either Eagle's basal medium (7) containing 10 per cent human serum, or as previously described (8), in a growth fluid composed of 40 per cent human serum and Hanks' balanced salt solution.

Viruses.—The viruses used were the prototype adenovirus strains, types 1 (9) and 4 (10). These agents were propagated by serial passage in HeLa cells and stock viral pools were prepared and stored by methods reported earlier (8).

Virus Infection of HeLa Cells.—Bottles and tubes of HeLa cell cultures were washed 2 to 3 times with Hanks' balanced salt solution; the cultures then were maintained during infection in a mixture containing 67.5 per cent Scherer's amino acid–vitamin mixture (6), 25 per cent tryptose phosphate broth, and 7.5 per cent chicken serum (11) (hereafter termed maintenance mixture). The amount of virus used for infection of cultures in bottles and the time which elapsed between infection and harvest varied among the experiments; these data are included in the description of experiments. Infectivity titrations were performed in HeLa cell culture tubes using 1:3.2 ($10^{-0.5}$) serial dilutions; the details of the methods have been given elsewhere (8). Employing this titration procedure it has been shown that a difference of 0.36 log units between 2 infectivity titers should occur by chance only once in 20 titrations (8).

Cell Counts.—One ml. of a suspension of intact HeLa cells or nuclei was centrifuged at 2500 R.P.M. for 10 minutes in an International type 1 model SB centrifuge and the supernate was discarded. The cells were resuspended in 1 ml. of versene (ethylenediaminetetraacetic acid, 1 mg./ml. normal saline solution, neutralized with $NaHCO_3$), incubated at 37°C. for 10 minutes, and mixed 30 times with a 1 ml. syringe through a 20 gauge needle. Counts were performed in a standard hemocytometer, using a phase-contrast microscope.

Complement Fixation Titrations.—The cytoplasmic and nuclear fractions of infected HeLa cells were satisfactory complement-fixing antigens and titrations of these antigens were performed using a standard complement fixation test (10). The serum from a patient with adenovirus complement-fixing antibody was heated at 56°C. for 30 minutes and diluted so that 4 units of antibody were contained in 0.25 ml. Serial 2-fold dilutions of the antigens in 0.25 ml. amounts and 2 full units of guinea pig complement in 0.5 ml. were then added to the tubes containing serum. After incubation at 37°C. for 2½ hours, 0.5 ml. of the hemolytic system, which consisted of 0.25 ml. of 3 per cent sheep cell suspension and 0.25 ml. of sheep cell amboceptor containing 3 units, was added and the test incubated at 37°C. for an additional 30 minutes. Veronal buffer containing 0.05 M $MgCl_2$ was employed throughout (12). The test was read after overnight refrigeration; titers are expressed as the final dilution of antigen, before addition of the hemolytic system, giving complete fixation.

Cell Fractionation.—A modification of a fractionation procedure involving the use of isotonic (0.25 M) and hypertonic (0.88 M) solutions of sucrose, described by Schneider and Hogeboom (13) was employed. The details of the method used are shown in a flow diagram in Fig. 1. HeLa cells were homogenized in a Sorvall omni-mixer. An International type 1 model SB centrifuge was used throughout the fractionation procedure. The 0.25 M and 0.88 M sucrose solutions were buffered to a pH of 7.5 with 0.05 M phosphate buffer, and chicken serum (5 per cent of final volume) was added to both solutions.

The number of whole cells or nuclei was determined before and after the cells were treated in the blender and in the nuclear fraction. At each step of the fractionation procedure aliquots of appropriate material were saved and stored at −25° to −28°C. until virus titrations were performed. Titrations were done directly on the supernates that contained no whole cells or

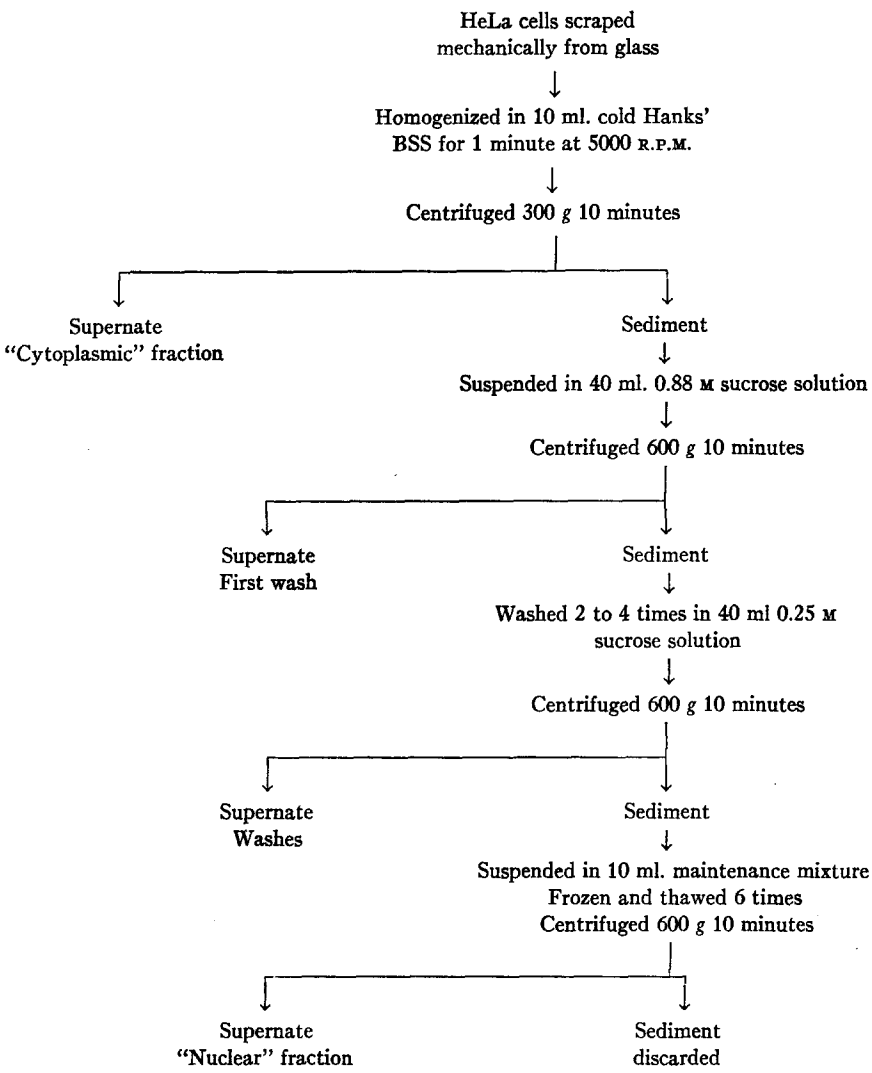

FIG. 1. Method of separating "nuclear" and "cytoplasmic" fractions of HeLa cells.

intact nuclei. The whole cells or intact nuclei in other specimens were disrupted by freezing and thawing 6 times or by ultrasonic vibration. Cellular debris was removed by centrifuging 600 *g* for 10 minutes and titrations were performed on the supernates.

The cells or nuclei in the various stages of the fractionation procedure were observed under the phase-contrast microscope. It should be noted that, in contrast to the clear detail of HeLa cells growing flat on glass, when these cells were removed from the glass surface they were rounded, making accurate observations of the cytoplasmic mass difficult.

EXPERIMENTAL RESULTS

Distribution of Infectious Type 4 Adenovirus within HeLa Cells.—The following experiment was performed to determine the intracellular localization of type 4 adenovirus in HeLa cells infected for 24 hours. This experiment is presented in detail because it is representative of other experiments described below and illustrates the methods used in determining the distribution of virus.

HeLa cells in one 32 ounce bottle were infected with $1.3 \times 10^{5.0}$ TCD_{50} of type 4 adenovirus. Six hours later, after a maximal amount of virus had been adsorbed to the cells, the maintenance mixture covering the cells was removed, the cells were washed gently once with 100 ml. Hanks' balanced salt solution containing 2 per cent chicken serum, and fresh maintenance mixture was added to the bottle. The infected cells were incubated for an additional 18 hours, the fluid removed and the cells fractionated into cytoplasmic and nuclear fractions as described under Materials and Methods (Fig. 1).

TABLE I

Distribution of Type 4 Adenovirus within HeLa Cells 24 Hours after Infection

Fraction	Quantity of virus		No. of cells or nuclei in fraction		
	Infectivity titer	Per cent of original specimen	Intact cells or nuclei	Disrupted nuclei	Per cent of original specimen
	log				
Original*	−3.69	100	524	0	100
Cytoplasmic	−3.75	100+	0	100	19‡
Nuclear	−2.75	11.4	148	0	28‡

* Infected HeLa cells before being subjected to any disrupting procedure.

‡ The failure of these figures to total 100 per cent was due to loss during washing.

The results of this experiment are shown in Table I. The cytoplasmic fraction contained the same amount of virus as was obtained from the whole cells, while only 11.4 per cent of the total measurable virus could be detected in the nuclear fraction. Because some nuclei were disrupted by the blender it was necessary to determine the number disrupted, thereby contributing nuclear contents to the cytoplasmic fraction, and also the number of nuclei remaining in the nuclear fraction. When these results are examined (Table I) it can be seen that, although the same amount of virus was detected in the cytoplasmic fraction as in the original cells, only 19 per cent of the nuclei had been disrupted. In contrast, 28 per cent of the nuclei remained intact and were contained in the nuclear fraction, while in this fraction only 11.4 per cent of the total virus could be isolated. It would appear, therefore, that, in this experiment, the larger amount of infectious virus was associated with the cytoplasmic fraction and that this cytoplasmic virus could not be accounted for by disruption of nuclei during the preparation of this fraction.

The cytological changes due to herpes simplex virus are located principally in the nucleus, although experiments by Gray and Scott (14) indicate that infectious virus is associated with the nucleus for only a short period of time. Therefore, it was of interest to determine whether the same series of events might not be true for adenovirus-infected cells. It has been shown that the period before the appearance of newly synthesized type 4 adenovirus in HeLa cells is inversely proportional to the amount of virus used to infect the cells (8). The "eclipse period" (elapsed time between viral inoculation and detection of newly formed virus) is 14 to 15 hours when maximal infecting doses of virus are used and the initial multiplication cycle is measured. Experiments were therefore performed to determine the distribution of virus in the cytoplasmic and nuclear fractions of HeLa cells during the early as well as late periods of infection (22 to 120 hours) (Table II). All experiments were similar except for the variation of the incubation period and the amount of virus used to infect the HeLa cells. In experiments with incubation periods of 24 hours or less, HeLa cell cultures were inoculated with $1.3 \times 10^{5.0}$ TCD_{50} of type 4 virus. Because previous experience had shown that this large inoculum of virus caused cytopathic changes of HeLa cell cultures in approximately 24 hours, a smaller inoculum ($1.3 \times 10^{3.0}$ TCD_{50}) was used for all experiments when infected cultures were incubated for 36 hours or longer. In Table II the percentage of total virus that was obtained from the separate fractions is compared with the percentage of nuclei which could have contributed virus to each fraction. At all time periods, more virus was detected in the cytoplasmic fraction than could be accounted for by the disrupted nuclei included. In contrast, the percentage of nuclei that remained intact and was contained in the nuclear fraction was greater than the percentage of virus found in that fraction. There was considerable variation between experiments in the percentage of nuclei disrupted by the blender and the percentage of intact nuclei remaining in the nuclear fraction. This was not related to the time of incubation of the HeLa cells and was thought to be due to variations inherent in the methods used to fractionate the cells. As with the experiment performed 24 hours after infection (Table I), these data indicate that more infectious type 4 adenovirus was detected in the cytoplasm of HeLa cells than in the nuclei. In addition, this distribution was not related to the period of incubation after infection of the cells.

Release of Virus from Nuclei in the Nuclear Fraction.—The nuclear fractions in the above experiments, although containing less virus than the cytoplasmic fractions, always showed significant amounts of infectious virus. It should be emphasized that the virus demonstrated in the nuclear fraction was obtained from nuclei disrupted by repeated freezing and thawing or by ultrasonic vibration, following repeated washing of nuclei to remove superficially adsorbed virus. This phenomenon can be illustrated best by presenting in more detail the results of the experiment performed on HeLa cells infected for 24 hours.

The infectivity titer of the original HeLa cells was $10^{-3.69}$ TCD_{50} and of the cytoplasmic fraction, $10^{-3.75}$ TCD_{50}. The titers of the first and fourth nuclear wash fluids were $10^{-2.35}$ TCD_{50} and $10^{-1.35}$ TCD_{50}, respectively. Following the last wash nuclei were frozen and thawed

TABLE II

Distribution of Type 4 Adenovirus in the Cytoplasmic and Nuclear Fractions of HeLa Cells Infected for Various Periods

Hrs. after inoculation*	Cytoplasmic fraction		Nuclear fraction	
	Virus‡	Disrupted nuclei included§	Virus‡	Intact nuclei‖
	per cent	*per cent*	*per cent*	*per cent*
22	ND¶	ND	4	18
24	100+	19	11	28
36	51	0	5	61
48	78	32	<1	5
	71	6	4	31
	100+	0	13	74
	49	9	9	75
	40	29	13	44
	100+	50	10	24
	100+	52	ND	ND
	100+	83	ND	ND
72	52	12	5	63
	89	37	ND	ND
120	100+	10	7	18
	68	31	1	18

* Cultures harvested at 22 and 24 hours infected with $1.3 \times 10^{5.0}$ TCD_{50} virus; those harvested 36 or more hours after inoculation were infected with $1.3 \times 10^{3.0}$ TCD_{50} virus.

‡ Per cent of total virus.

§ $\frac{\text{Total cells} - \text{nuclei after blender}}{\text{Total cells}} \times 100.$

‖ $\frac{\text{Intact nuclei after repeated washes}}{\text{Total cells}} \times 100.$

¶ Not done.

6 times. The infectivity titer of the supernate from this suspension was found to be $10^{-2.75}$ TCD_{50}, a 25-fold increase in virus over the last wash. Similar data were obtained in other experiments and are presented in Table III. In this table the infectivity titrations of the nuclear fractions are compared with those of the last wash before the nuclei were disrupted. Significant amounts of virus were released in all instances by procedures that disrupted the nuclei, the values varying from $10^{0.4}$ to $10^{1.75}$ TCD_{50}.

These data suggest that the nucleus, as well as the cytoplasm, contains infectious virus. It is possible, however, that the cytoplasm of the cells was not completely removed from the nuclei, and procedures that disrupted the nuclei also released cytoplasmic virus. An effort was made to clarify this point by microscopic examination of the nuclear fraction. Fresh preparations were examined by phase-contrast microscopy and stained preparations were observed under the light microscope. In all specimens examined, fragments or tags of cytoplasm were attached to many nuclei. Therefore, although it is a possibility that the virus released into the nuclear fraction was from these

TABLE III

Comparison of the Virus Infectivity Titers of the Nuclear Fraction and the Supernate from the Last Wash before Disruption of the Nuclei

Hrs. after infecting HeLa Cells*	Virus infectivity titer	
	Last wash	Nuclear fraction‡
	log	*log*
22	−0.85	−2.0
24	−1.35	−2.75
36	−1.85	−3.0
48	0	−0.5
	−1.6	−2.75
	−1.6	−2.5
	−1.85	−3.0
	−2.1	−3.5
	−2.1	−3.25
72	−2.6	−3.0
120	−1.25	−2.75
	−0.75	−2.5

* Type 4 adenovirus used in all experiments.

‡ Nuclei disrupted by repeated freezing and thawing or ultrasonic vibration.

cytoplasmic tags, the mass of cytoplasmic material observed appeared to be too small to account for the quantity of infectious virus released.

Distribution of Type 1 Adenovirus within HeLa Cells.—Previous studies have shown that types of adenoviruses differ in the latent period of the initial multiplication cycle, in the quantitative relationship of virus with homologous type-specific antibody, in the yield of virus per HeLa cell, and in cytological changes produced in HeLa cells (8, 15). Types 3 and 4 have similar characteristics but differ from types 1 and 2 which resemble each other. To determine whether these groups of adenoviruses differed also in intracellular localization of infectious virus, the type 1 agent was studied. Table IV shows the distribution of this adenovirus in the cytoplasmic and nuclear fractions of HeLa cells infected for various periods. In all experiments the cytoplasmic fraction con-

tained as much or more virus than could be accounted for by the number of disrupted nuclei included in that fraction. In the experiments on cells fractionated 48 and 96 hours after inoculation, the percentage of nuclei in the nuclear fraction was slightly greater than the percentage of virus found in

TABLE IV

Distribution of Type 1 Adenovirus in the Cytoplasmic and Nuclear Fractions of HeLa Cells Infected for Various Periods

Hrs. after inoculation	Cytoplasmic fraction		Nuclear fraction	
	Virus*	Disrupted nuclei included‡	Virus*	Intact nuclei§
	per cent	*per cent*	*per cent*	*per cent*
24	100	48	56	23
48	56	38	18	34
72	100+	55	ND‖	ND
96	100	50	18	25

* Per cent of total virus.

‡ $\frac{\text{Total cells} - \text{nuclei after blender}}{\text{total cells}} \times 100.$

§ $\frac{\text{Intact nuclei after repeated washes}}{\text{total cells}} \times 100.$

‖ Not done.

TABLE V

Comparison of the Type 1 Adenovirus Infectivity Titers of the Nuclear Fraction and the Supernate from the Last Washing before Disruption of the Nuclei

Hrs. after inoculating HeLa cells	Infectivity titer	
	Last washing	"Nuclear" fractions*
	log	*log*
24	−3.35	−4.75
48	−4.85	−5.75
96	−4.35	−5.0

* Nuclei disrupted by repeated freezing and thawing or sonic vibration.

that fraction. At 24 hours, however, a higher percentage of virus was located in the nuclear fraction than could be accounted for by the number of nuclei in the fraction, thus suggesting that the virus in the cytoplasmic fraction in this experiment could have been derived from disrupted nuclei. The results of this experiment, and the small differences shown between the amounts of virus in the cytoplasmic and nuclear fractions in the other experiments, suggest the possibility that type 1 differs from type 4 in the localization of infectious virus.

The release of type 1 adenovirus by the disruption of nuclei is shown in Table V. The nuclear fraction in all experiments contained significantly more virus than did the last nuclear wash fluid. As in the experiments with the type 4 virus, these data suggest that the nucleus contains infectious virus, but the possibility that the virus in the nuclear fraction was from cytoplasm attached to nuclei could not be excluded.

Effect of the Fractionation Procedure on Normal and Infected HeLa Cells.— Because electron and light microscopy have shown principally nuclear changes in adenovirus-infected HeLa cells, and, in contrast, the studies reported here suggest that the greatest concentration of infectious adenovirus is in cytoplasmic fractions of these cells, an effort was made to determine whether this discrepancy could be explained by the selective disruption of infected cells

TABLE VI

Comparison of the Effect of the Blender on Normal and Infected HeLa Cells

HeLa cells	Cell count before blender	Cell count after blender*	Per cent cells disrupted in blender
	× 10^3 *per ml.*	× 10^3 *per ml.*	
Normal	868	482	45
Infected‡	801	459	38
Normal	903	553	39
Infected	754	523	31
Normal	610	335	45
Infected	360	286	21

* Sorvall blender 5000 R.P.M. 1 minute.

‡ Type 1 adenovirus.

during the fractionation procedure. If infected cells were more easily disrupted by the blender than were normal cells, the cytoplasmic fraction might contain virus that was nuclear in origin. Experiments were first designed to determine whether the blender disrupted a higher percentage of HeLa cells in a culture infected with an adenovirus than in an uninfected culture. The results of 3 such experiments are shown in Table VI, in which cell counts before and after the blender are compared in uninfected and type 1 adenovirus-infected HeLa cell cultures. Type 1 virus was employed because under the experimental conditions employed and time intervals used a higher percentage of cells was infected with type 1 than with type 4 virus. In all experiments the percentage of normal cells lost during this procedure was of the same order of magnitude in the uninfected and infected cultures. The differences noted are not large and are not thought to be significant, but do indicate that there was not marked selective disruption of infected cells.

The above experiments did not eliminate the possibility that infectious adenovirus was produced by a relatively small percentage of infected HeLa cells and that it was these cells that were destroyed. Cytologic studies (4) suggested this possibility because the changes noted by light microscopy were progressive and could be related to time after infection. Attempts were therefore made to determine the percentage of normal and infected cells that was present at different stages of the fractionation procedure. These experiments were only partially successful. It was not possible to make quantitative studies from smears of the cells or nuclei because large numbers of these washed off during the fixing and staining procedures. This difficulty

TABLE VII

Quantity of Type 4 Adenovirus in Cytoplasmic and Nuclear Fractions as Measured by Infectivity and Complement Fixation Titrations

Fraction	Infectivity titer	Complement fixation antigen titer	Ratio cytoplasmic/nuclear	
			Infectivity	Complement fixation
	log		*log*	*log*
Cytoplasmic	−4.25	1:64	1.5	1.2
Nuclear	−2.75	1:4		
Cytoplasmic	−4.25	1:256 or more	1.75	1.8
Nuclear	−2.50	1:4		
Cytoplasmic	−4.00	1:32	1.25	0.9
Nuclear	−2.75	1:4		
Cytoplasmic	−3.50	1:32	1.0	0.9
Nuclear	−2.50	1:4		

was overcome by suspending the cells in a plasma clot, from which sections were cut and stained with hematoxylin and eosin. This procedure, however, caused distortion of nuclei, making observations on the status of infection difficult. Nevertheless, in spite of this difficulty, it was possible to identify infected cells in the majority of instances and an attempt was made by this method to determine the percentage of infected cells in several experiments. In no instance was an excessive number of infected cells disrupted, thus confirming the earlier experiments which indicated that infected cells were not selectively destroyed by the blender.

Comparison of Infectivity and Complement Fixation Titrations in Cytoplasmic and Nuclear Fractions.—It has been demonstrated that an antigen is produced in adenovirus-infected HeLa cells that will fix complement in the presence of specific antibody (9, 16). This antigen could be separated from the infectious

virus by high speed centrifugation, was common to the large group of adenoviruses, and was not type-specific. Because light and electron microscopy of adenovirus-infected HeLa cells showed major nuclear changes while the present studies showed the bulk of infectious virus in the cytoplasm, it was of interest to determine whether the nuclear fraction contained more complement-fixing antigen than did the cytoplasmic fraction. If this were the case, it would suggest that the microscopic nuclear changes did not indicate the presence of mature, infectious virus. Complement fixation titrations were therefore performed on nuclear and cytoplasmic fractions of HeLa cells infected with type 4 adenovirus. The results of these experiments are shown in Table VII, which also includes the infectivity titrations of the same fractions. When the ratios of cytoplasmic and nuclear virus are compared, using both infectivity and complement fixation titrations, it can be seen that the resulting figures are remarkably similar. These results indicate that infectious virus and viral antigen, as measured by complement fixation, are similarly distributed between cytoplasm and nucleus of the infected cell. Moreover, the data suggest that localization of the major portion of the infectious virus in the cytoplasm does not signify that most viral particles attain infectivity at this site, but rather that the viral material is more easily detected when it is located in the cytoplasm.

DISCUSSION

The results of this study indicate that when type 4 adenovirus-infected HeLa cells were separated into cytoplasmic and nuclear fractions by mechanical disruption and differential centrifugation, the major portion of the infectious virus and complement-fixing antigen could be isolated from the cytoplasmic fraction. The data obtained from experiments performed with type 1 adenovirus-infected cells were similar to those obtained with type 4 virus, but the quantity of virus measured in the cytoplasmic fraction could have been contributed by the number of nuclei disrupted by the fractionation procedures. The difference between the studies done with the 2 types of viruses suggests that type 1 may differ from type 4 in the localization of the major portion of the detectable infectious virus.

The finding that the majority of infectious virus was in the cytoplasmic fraction was not anticipated because it has been shown by light (4, 17), electron (1–3), and phase-contrast (5) microscopy that the nucleus is the site of the major alterations in adenovirus-infected HeLa cells. Electron microscopic investigations have demonstrated crystalline-arrays of virus-like particles within the nucleus; virus-like particles have been found in the cytoplasm only in small numbers in cells with disrupted nuclear membranes. Furthermore, fluorescent antibody studies have shown that the majority of specific viral antigen is intranuclear (5). Various hypotheses were therefore sought to explain the data obtained in the investigations described in the present paper.

The consistently reported low infectivity titers relative to the large number of virus-like particles suggest that only a minute amount of viral material synthesized is infectious and raises the possibility that infectious properties may not be acquired by viral particles until they emerge into the cytoplasm. With this hypothesis, the detection of infectious virus in the nuclear fraction could be explained by the presence of virus in cytoplasmic tags attached to nuclei. The relatively few infectious particles in the cytoplasm would be difficult to demonstrate by electron microscopy and might not be detected by fluorescent antibody techniques. The complement fixation studies, which indicate that most complement-fixing antigen is also isolated in the cytoplasmic fraction, however, are not compatible with this hypothesis.

Perhaps a more likely interpretation is that intranuclear viral particles are tightly bound in crystalline-lattice arrays, and that such aggregates, although composed of a large number of viral particles, may act as single infectious units. In contrast, the virus which has moved into the cytoplasm is no longer in aggregated form. Therefore, although the actual viral mass in the cytoplasmic fraction may be smaller, measurement by either infectivity or complement-fixation titrations erroneously implies the presence of more virus. This hypothesis may also explain the possible difference between types 1 and 4 viruses. It has been demonstrated that type 1 viral particles are less frequently bound into crystalline-like arrays than are type 4 particles (2); a larger number of individual particles in the nuclei of type 1–infected cells would therefore be measured as infectious units.

Many technical difficulties are encountered in fractionation procedures for the isolation of cellular components and the results of experiments in which these procedures are employed should be interpreted with caution. The technique involving mechanical disruption of cells and repeated washings with sucrose solutions was adopted after preliminary studies with adenovirus-infected HeLa cells showed that this procedure gave better results than other methods tested. Several other techniques were discarded when it was found that the yield of nuclei was too low or that adenovirus was inactivated during the procedure (18–21). The method employed was originally described by Schneider and Hogeboom (13) and used successfully by Ackermann and Kurtz (22) and Gray and Scott (14) in studies of the intracellular localization of herpes simplex virus in the liver of the mouse or chick embryo. Although this technique gave the most satisfactory results of any procedure tried, difficulties inherent in the method employed gave rise to concern with the interpretation of results; these will be mentioned. The technique used to disrupt cells so that nuclear and cytoplasmic fractions could be obtained subjected cells to harsh treatment, and it was inevitable that many nuclei were disrupted thereby contributing their contents to the cytoplasmic fraction. Experiments performed to determine whether infected nuclei were selectively disrupted

indicated that this was not the case. It should be noted, however, that the methods available for determining selective disruption were relatively crude and might not have detected the disruption of the cells containing infectious virus if the infectious virus was produced by a relatively small percentage of cells. The possibility that nuclear contents were leached from the nucleus and were included in the cytoplasmic fraction was also considered. In experiments with polioviruses (23) and herpes simplex viruses (14), fractionation procedures similar to the one employed in the studies described here have showed that the greatest quantity of these agents at some time during the multiplication cycle was in the nuclear fraction, results which suggested that leaching did not occur. Whether infectious adenovirus was located only in the nucleus and was leached out into the cytoplasmic fraction could not be determined, but from results reported with other viruses, this seems unlikely. In addition, the release of only a small percentage of the total detectable virus from the intact infected cell into the tissue culture fluid is further evidence to suggest that the result described probably is not caused by leaching (8).

A third factor to be considered was whether the techniques used completely dissociated the cytoplasm from the nucleus. This consideration was important because small amounts of infectious virus were regularly released from disrupted nuclei which had been washed repeatedly. This suggested either that the nucleus contained infectious virus or that the virus was in the fragments of cytoplasm that had not been removed from the nucleus and was therefore released when the nucleus was broken. Phase-contrast microscopy indicated that fragments of cytoplasm attached to many nuclei were so small that it seemed doubtful that they could have contributed the quantity of virus in the nuclear fraction, thus suggesting that the nucleus contained infectious virus. Therefore, after consideration of the technical difficulties encountered in the fractionation procedure employed, it did not seem probable that these difficulties could explain the results obtained.

The exact sequence of the development of infectious adenovirus is still not known. It is clear that the majority of infectious virus which can be measured is associated with the cytoplasm. Whether this is due to the development of the infectious property in the cytoplasm or to the marked aggregation of viral particles within the nucleus has not been determined. Whatever the true explanation may be, these experiments have suggested a reason for the small quantities of infectious virus obtained from cells apparently plethoric with viral materials.

SUMMARY

HeLa cells infected with types 1 or 4 adenovirus were separated into cytoplasmic and nuclear fractions by mechanical disruption and differential centrifugation and the quantity of infectious virus in each was determined. The

results showed that the majority of infectious virus of both types could be isolated in the cytoplasmic fraction. It was not possible to explain the large amount of type 4 virus in the cytoplasmic fraction by the number of nuclei disrupted in the fractionation procedure, but the amount of type 1 virus in the cytoplasmic fraction could have been contributed by disrupted nuclei. This suggested that there might be a basic difference in the intracellular formation of the two types of virus. The intracellular distribution of complement-fixing antigen was similar to that of infectious virus in type 4-infected cells. Technical difficulties, inherent in cellular fractionation studies, were encountered but did not appear to explain the results obtained.

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