An Immunofluorescence Assay for Studying Replication of Adeno-Satellite Virus

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(Accepted 5 December 1966)

SUMMARY

The simian adeno-satellite virus apparently cannot multiply unless host cells are co-infected with a 'helper' adenovirus. Following simultaneous infection of green monkey kidney cells with simian adenovirus (SV 15) and its satellite, satellite antigen was first detected by immunofluorescence in the nucleus 10 to 12 hr after inoculation, while adenovirus antigen was first detected at 16 hr. A single cycle of growth for satellite virus was completed in about 24 to 48 hr. Inoculation of satellite-free adenovirus from 10 to 15 hr before inoculation with satellite proved to be the most efficacious time for shortening the latent period of satellite. Satellite antigen could then be detected as early as 4 hr after inoculation. These results indicate that events in the adenovirus replication cycle must proceed for 10 to 12 hr before satellite can be synthesized.

An infectivity titration of satellite based on immunofluorescence was developed. The percentage of fluorescent cells in standard monolayers was determined and the infectious units were calculated from the dilution infecting 1% of cells. Most satellite preparations had titres of $10^8$ to $10^9$ infectious units/ml, with one infectious unit equivalent to 30 to 100 particles.

INTRODUCTION

There have been a number of reports concerning the 200 Å particles containing DNA which have been found in preparations of a number of adenoviruses of man and lower animals (Atchison, Casto & Hammon, 1965; Hoggan, Blacklow & Rowe, 1966; Mayor et al. 1965; Melnick et al. 1965). As recently pointed out (Crawford, 1966; Mayor & Melnick, 1966), these particles appear to be morphologically similar to the H1, H3, RV, X14 and MM viruses of rodents. However, unlike the rodent viruses which are capable of continuous replication in susceptible host cells, the particles found in primate adenovirus harvests appear to be defective in that their multiplication is dependent on co-infection of the host cells with a fully competent 'helper' adenovirus (Atchison et al. 1965; Hoggan et al. 1966; Parks et al. 1967). Atchison et al. (1965) suggested that particles be called adeno-associated viruses; but as they behave more like the previously described satellite viruses of plants, than like Rous-associated virus (Haselkorn, 1965; Mayor & Melnick, 1966) or SV 40 which has often proved to adeno-associated, the particles have been called 'adeno-satellite' viruses in this laboratory.

The only assay systems so far available for detecting the satellite have been complement fixation and electron microscopic particle counting (Atchison et al. 1965;
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Hoggan et al. 1966; Parks et al. 1967). Accurate and reproducible counts may be obtained with preparations containing relatively small numbers of satellite particles (limit, about $10^7$/ml.) but the technique can only detect morphological entities and cannot discriminate between particles on the basis of viability. We have developed an immunofluorescent infectivity titration which is capable of detecting viable satellite, similar to the measurement of infectivity by testing for complement fixing antigen cultures inoculated with different dilutions of virus (Atchison et al. 1965; Hoggan et al. 1966). Using the immunofluorescence assay we have followed the increase of infectivity of the satellite in the presence of known amounts of helper adenovirus SV 15. The effects of pre-treating the cells with competent adenovirus before inoculation with satellite, simultaneous inoculation with both viral agents, and inoculation with adenovirus after satellite were studied. The sensitivity of different cell cultures for assay purposes was tested in primary and secondary cultures of African green monkey kidney, and the BSC-1 stable line of green monkey kidney.

METHODS

Cells. Primary African green monkey (GMK) monolayer cell cultures and primary rhesus kidney cells were grown in M-H medium and maintained in M-E medium as previously described (Melnick, 1956). The BSC-1 continuous cell line (Hopps et al. 1963) was used in some experiments. The latter cell lines were grown in Eagle medium supplemented with 10% (v/v) calf serum and maintained in the same medium with 2% (v/v) serum.

Viruses. The SV 15 virus seed, T7MK 3518, was obtained from Dr W. McD. Hammon, University of Pittsburgh, and was propagated several times in rhesus and green monkey kidney cultures before use. Upon electron microscopy, this passage material was found to contain in addition to the SV 15 adenoviruses, satellite virus particles subsequently identified as type 4 (W. P. Parks, private communication). Consequently this seed will be called SV 15(4), using the nomenclature suggested by Hoggan et al. (1966). An SV 15 seed, free of satellite virus was prepared from the SV 15(4) material by three plaque purifications at limiting dilutions on GMK monolayers. This stock has continued free of satellite virus on many subsequent passages in GMK cells; it will therefore be called SV 15(0).

Virus stocks were grown in primary GMK cells in 16-oz bottles from both SV 15(4) preparations and from plaque-purified SV 15(0) seed. The satellite virus was prepared by equilibrium density gradient centrifugation as previously described (Mayor et al. 1965). At equilibrium there were two visible bands in the ultracentrifuge tube. The upper band (1.34 g./cm$^3$) contained adenovirus and some satellite particles (mostly empty); the lower band (1.42 g./cm$^3$) contained only satellite as determined by electron microscopy. This lower satellite band was dialysed against buffered saline and immediately before inoculation it was heated for 15 min. at 60° to inactivate any viable adenovirus possibly present (Atchison et al. 1965; Hoggan et al. 1966).

Growth of satellite and adenovirus. Growth of satellite in the presence of helper adenovirus was studied in Leighton tube cultures following simultaneous infection with both agents; in cells pretreated with satellite and subsequently inoculated with SV 15(0); and in cells pretreated with SV 15(0) and later inoculated with satellite virus. The development of satellite antigen was followed by the immunofluorescence
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Assay method described below. The SV 15 adenovirus was assayed by titration in GMK cultures using a 50% cytopathic endpoint (TCD 50).

Coverslip cultures were inoculated with 0.1 ml. of virus and allowed to stand for 2 hr at 37°. One ml. of maintenance fluid was added, and incubation continued as described. With the simultaneous mixed infections the concentration of the inoculum was adjusted so that the final concentration of each virus was the same as in the singly infected cultures. Inocula contained input multiplicities of the order of 100 particles per cell for the satellite virus and 100 TCD 50 per cell for adenovirus. Cultures were harvested at regular intervals for fluorescent antibody staining, complement fixation, and particle counting.

Antisera against purified satellite and against SV 15(0) were prepared in rabbits using, as antigens, viruses purified by double banding in CsCl (1.42 g./cm.\(^3\) for the satellite and 1.34 g./cm.\(^3\) for adenovirus). These sera were made available by Mr Wade Parks (Parks et al. 1967).

**Fluorescent antibody procedures.** Crude globulin fractions were prepared from each serum by precipitation with half-saturated (NH\(_4\))\(_2\)SO\(_4\) solution and labelled with fluorescein isothiocyanate by methods previously described (Mayor, 1961) with slight modifications. The reagent mixtures containing antibody globulin, carbonate buffer at pH 9.0, and fluorescein isothiocyanate, were allowed to react for 6 to 8 hr at 4°; immediately afterwards they were passed through Sephadex G 50 columns to eliminate unbound dye. Each labelled conjugate was adsorbed with acetone-dried monkey liver powder before use. For immunofluorescence, infected and control coverslips were dried in air, fixed with acetone at room temperature for 10 min., and again dried in air before staining with the appropriate conjugate for about one hr at room temperature. The highest dilution of fluorescent antibody capable of giving a positive reaction was determined by preliminary staining tests. Usually from 2 to 4 times more concentrated reagents were used in routine staining than indicated by end point dilution; anti-satellite virus serum was used at a dilution of 1/8 and anti-adenovirus serum at 1/16. Mayor (1961) described details of fluorescence microscopy and photomicrography used in this laboratory.

**Titration method for satellite infectivity by immunofluorescence.** Serial tenfold dilutions of the material for testing were made, and 0.2 ml. of each dilution was inoculated to primary GMK monolayers grown on coverslips (11 mm. \(\times\) 22 mm.) in Leighton tubes. At least two cultures, each containing about 300,000 cells, were inoculated with each dilution of virus. At the same time, 0.1 ml. of a SV 15(0) helper adenovirus was added to each culture at an input multiplicity of about 40 TCD 50/cell. The inoculated cultures were allowed to incubate for 2 hr at 37° for adsorption; afterwards, 1 ml. of maintenance medium was added, and the inoculated cultures were incubated for an additional 18 hr. Cells developing specific satellite antigen were counted in randomly selected areas, and the number in the whole cell population was determined for each culture (Table 1). If the infected monolayers are stained carefully over the entire area, as many as 100,000 cells can be examined by fluorescence microscopy on each coverslip.

**Growth of satellite virus in GMK cells.** To study the time course of replication of satellite virus, experiments were designed as follows. Primary GMK cultures containing \(3 \times 10^5\) cells were inoculated with SV 15(0) and satellite in two ways. One group was infected with both viruses at the same time (mixed infection) and the other inoculated
with satellite 12 hr after SV 15(0) (superinfection with satellite). Viruses were inoculated at the same input multiplicities as above. At 12, 16, 20, 24, 36 and 48 hr after the mixed infection, and at 4, 8, 12, 24 and 36 hr after the superinfection with satellite, four inoculated cultures from each group were harvested. Two of them were frozen at once. The fluids from two cultures were pooled to test the extracellular infectivity, while the remaining two were frozen and thawed three times to provide material for measurement of total infectivity. The infectivity titration was done by the immunofluorescent assay method as described above and repeated at least twice for each sample.

**RESULTS**

The immunofluorescence assay

Figure 1 shows the results of plotting the percentage of cells against the dilution of inoculum; two lots of primary GMK cells and one lot of BSC-1 cells were used in an assay and fluorescent cells showing satellite antigen were counted 20 hr after inoculation. There was a linear relationship within the range of 0.01% to 30% fluorescent cells (Fig. 1). For precision, therefore, it is necessary to estimate the number of fluorescent cells in the region where approximately 1% of the cells show specific fluorescence. Fluorescent infectivity units (f.i.u.)/ml. of the material can be calculated from the average number of fluorescent cells in the monolayer at the end point and the dilution of the inoculum. If the volume of the inoculum is 0.2 ml. then f.i.u./ml. = reciprocal of dilution × average number of fluorescent cells × 5 (Table 1).

**Table 1. Calculation of fluorescent infectious units (f.i.u.) of adeno-satellite virus in SV 15(4) lot 4**

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Total no. cells counted</th>
<th>No. of fields examined</th>
<th>Average no. fluorescent cells/field*</th>
<th>Percentage of fluorescent cells</th>
<th>No. fluorescent cells in Leighton tube†</th>
<th>F.i.u./ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁻²</td>
<td>480</td>
<td>2</td>
<td>240-0</td>
<td>60-0</td>
<td>180,000</td>
<td>9·0 x 10⁷</td>
</tr>
<tr>
<td>10⁻³</td>
<td>315</td>
<td>3</td>
<td>105-0</td>
<td>26-0</td>
<td>76,000</td>
<td>3·9 x 10⁶</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>108</td>
<td>10</td>
<td>10-8</td>
<td>2-7</td>
<td>8,000</td>
<td>4·0 x 10⁵</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>128</td>
<td>10</td>
<td>12-8</td>
<td>3-2</td>
<td>9,600</td>
<td>4·8 x 10⁵</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>82</td>
<td>40</td>
<td>2·5</td>
<td>0·5</td>
<td>1,540</td>
<td>7·7 x 10⁵</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>34</td>
<td>40</td>
<td>0·85</td>
<td>0·2</td>
<td>640</td>
<td>3·2 x 10⁴</td>
</tr>
<tr>
<td>10⁻⁸</td>
<td>36</td>
<td>220</td>
<td>0·16</td>
<td>0·04</td>
<td>120</td>
<td>6·0 x 10³</td>
</tr>
<tr>
<td>10⁻⁹</td>
<td>24</td>
<td>220</td>
<td>0·11</td>
<td>0·027</td>
<td>80</td>
<td>4·0 x 10³</td>
</tr>
</tbody>
</table>

Average‡: 4·7 x 10⁸

* Average of 400 cells per microscopic field (magnification, ×120).
† Total number of cells per Leighton tube, 3 x 10⁵.
‡ Average f.i.u. obtained from 7 coverslips showing a range of 0.01% to 30% of fluorescent cells.

F.i.u./ml. can be calculated from a consideration of the total number of cells per Leighton tube and the dilution of virus that produces fluorescence in 1% of the cells as determined from curves such as that shown in Fig. 1. In this example, the 1% end point dilution in GMK lot 1 was 10⁻⁴, and the total number of cells in the culture was 3 x 10⁵. If the volume of the inoculum is also 0.2 ml. then,

\[
\text{f.i.u./ml.} = 10^{4} \times 0.01 \times 3 \times 10^{5} \times 5 = 4.8 \times 10^{8}
\]

Although the f.i.u. titre was 4.8 x 10⁸ per ml., the level of detectability was about 10³
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to $10^4$ f.i.u./ml. Thus, the virus stock could be diluted to $10^{-4}$ to $10^{-5}$ and satisfactory titres could be obtained.

Sensitivity of cells used for the assay

The infectious titres of the satellite virus given by the immunofluorescence assay varied with the type of cells used. Variation in titre was dependent upon the sensitivity of the cells both to satellite and to SV 15 and perhaps upon the interference that is sometimes found between the two viral agents (Atchison et al. 1965; Hoggan et al. 1966; Parks et al. 1967). The titres of satellite infectivity were highest on primary GMK cells (Table 2). Primary rhesus kidney cells gave infectious titres about one-tenth of those obtained in primary GMK cells for identical specimens. Secondary cell cultures were less sensitive than primary ones. The BSC-1 cells were consistently less sensitive than primary GMK cells as shown by satellite infectious titres of identical materials. Different lots of primary GMK cells also showed some variations in sensitivity (Table 2 and Fig. 1).

Reproducibility of the immunofluorescent assay

Reproducibility of the assay was tested by repeated titration of two virus stocks in different lots of primary GMK cells (Table 2). The total variation in titre was not greater than tenfold.

Sampling errors due to difficulties in determining an accurate percentage of infected cells.
Fig. 2. Growth curves of adenovirus (SV15(0)) antigen and satellite (ASV) antigen as determined by immunofluorescence. Satellite and adenovirus were inoculated together at time zero. Cultures were assayed for both antigens at regular time intervals thereafter, as indicated.

Table 2. Titration of adeno-satellite infectivity by immunofluorescence

<table>
<thead>
<tr>
<th>Satellite lot numbers</th>
<th>Green monkey kidney cells</th>
<th>Rhesus monkey kidney cells</th>
<th>BSC-1 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Primary cells</td>
<td>Secondary cells</td>
<td>Primary cells</td>
</tr>
<tr>
<td>Lot 1</td>
<td>$8 \times 10^7$ A*</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Lot 2</td>
<td>$2 \times 10^6$ B</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Lot 3</td>
<td>$3 \times 10^6$ C</td>
<td>$8 \times 10^6$ J</td>
<td>$3 \times 10^6$ K</td>
</tr>
<tr>
<td>Lot 4</td>
<td>$5 \times 10^5$ E</td>
<td>$4 \times 10^5$ J</td>
<td>$1 \times 10^6$ K</td>
</tr>
<tr>
<td>Lot 5</td>
<td>$2 \times 10^5$ F</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Lot 7</td>
<td>$3 \times 10^4$ G</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Lot 10</td>
<td>$4 \times 10^4$ H</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Letters refer to different batches of cells.

cells by the present counting method are less than tenfold (Table 2). The accuracy of the titration is, of course, considerably less than standard plaque counting methods for non-defective viruses.
The growth cycles of satellite and its adenovirus helper as determined by immunofluorescence

The growth cycles of satellite and adenovirus helper, when followed by immunofluorescent assay, did not differ significantly from the results obtained with the particle counting techniques (Fig. 2) (Parks et al. 1967). Two sets of experiments were done using primary GMK cells. One was designed to follow the time course of satellite and helper adenovirus replication after simultaneous mixed infection, and after superinfection with SV 15(0) of cells which had been infected with the satellite 48 hr previously. (Fig. 2, Expt 1). The other set of experiments was designed to follow the growth curve of satellite at closer time intervals after superinfection with the SV 15(0) helper (Fig. 2, Expt 2).

![Graph](image)

Fig. 3. Growth cycle of satellite (ASV) antigen when adenovirus was inoculated 0 (●), 4 (△), 8 (▲), 12 (□) and 16 (○) hr previously. Satellite antigen was assayed by immunofluorescence at 2 hr intervals after satellite inoculation.

The satellite antigen was first detected in the nucleus of the inoculated cells 12 hr after addition of the helper adenovirus. The frequency of fluorescent cells increased gradually, and reached its maximal level (50 to 90%) 48 hr after SV 15(0) inoculation. No satellite antigen was detectable in the cells when helper adenovirus was not added. The appearance of adenovirus antigen in the nucleus was usually 4 to 8 hr later than that of the satellite virus.

Alteration of the growth cycle of satellite by previous infection with helper adenovirus

In contrast to the previous experiments in which helper virus was added together with and after the satellite virus, in the next experiments helper virus was inoculated first and the satellite virus at various times afterwards, in order to clarify which step in the growth cycle of adenovirus was most effective in helping the replication of the satellite.
Helper adenovirus was inoculated into GMK cell monolayers on coverslips at a high input multiplicity (40 TCD 50/cell); and at 0, 4, 8, 12 and 16 hr after the inoculation the cell cultures were superinfected with the satellite at a multiplicity of about 100 particles per cell. At frequent intervals after the addition of satellite virus, the coverslips were harvested and the percentage of cells developing specific satellite antigen was determined on each coverslip by immunofluorescence (Fig. 3, 4). After simultaneous infection with satellite and adenovirus, the satellite antigen was first detected 10 hr after inoculation in a small number of nuclei (0.02% of the cells); and a growth curve similar to that in Fig. 2 was obtained. However, the longer the interval between SV 15(0) and satellite inoculation, the shorter was the eclipse period for the satellite virus. When the interval between SV 15(0) and satellite inoculation was 4 hr, the latent period of the satellite virus was about 8 hr. With an 8 hr interval, the latent period was shortened to 6 hr. Finally in the case of a 12 to 16 hr interval between virus infections, the latent period was as short as 4 hr (Fig. 4). The growth curve for the satellite virus when it was added at a longer interval after SV 15(0) tended to rise more rapidly (Fig. 3). These results indicate that events in the adenovirus replication cycle must proceed for 10 hr or more before satellite virus can be synthesized; however, when cells are primed by adenovirus for the multiplication of the satellite virus, its latent period is about 4 hr.

**Growth of satellite virus infectivity in GMK cells**

Although the growth curve of the total satellite virus began to rise about 12 hr after the mixed infection and reached its maximum at 24 hr, the extracellular infectivity did not begin to rise until 16 to 20 hr after the infection, indicating a release time of at least 4 hr for satellite virus. In cells superinfected with satellite, the total infectivity rose rapidly from 4 to 8 hr after superinfection while the growth curve of free virus was again delayed about 4 hr (Fig. 5).
DISCUSSION

The infectivity of the defective adeno-satellite virus can be assayed directly in a 24 hr test by means of immuno-fluorescence. In Leighton tubes containing about 300,000 cells, it is possible to identify as few as 10 immuno-fluorescent cells, although for convenience and accuracy about 3000 such cells in the culture are preferable. When 1 % of cells fluoresce, only 10 random fields, each containing 400 cells, need be counted. Thus for maximum sensitivity and accuracy, a lower limit of about 10,000 f.i.u./ml. must be present. However, under such conditions, it must be emphasized that the variation in infectivity is three- to tenfold.

The use of immuno-fluorescence to titrate viruses in systems in which the virus fails to produce its own specific cytopathology or in which cytopathology is not readily followed or is slow in developing is not new. Philipson (1961) reported a fluorescent cell-counting technique for assay of adenovirus which was equal in sensitivity and much faster than the standard plaque method. Hinuma, et al. (1963) have used the complement method of immuno-fluorescence to assay parainfluenza 1 (SENDAI) virus. Measles (Rapp, et al. 1959), cytomegalovirus (Goodheart & Jaross, 1963; Rapp,
Rasmussen & Benyesh-Melnick, 1963), and rubella (Brown et al. 1964), have been also assayed by immunofluorescence. Usually, fluorescent foci, i.e. groups of cells produced by continuous cycles of replication from one original infectious unit, are observed. In such a system it may take several days before a focus capable of giving visible fluorescence is developed. Our method of assay for the satellite virus is a direct and rapid procedure, since single cells containing satellite antigen can be identified and observed after a single cycle of growth. Recently, Altstein (in Moscow, personal communication) and Goldblum (in Jerusalem, personal communication) have used the ability of SV 40 to induce immunofluorescent tumour antigen as a 24 hr assay for infectious papovavirus particles.

In the immunofluorescence technique for the satellite virus, it is important that the inoculated cultures are harvested before secondary spread of the virus, that is, at the end of a single cycle of growth (Fig. 2). This restriction is imposed by virtue of the defective nature of the satellite. Under present conditions for growth, the satellite virus can be replicated only in cells also infected with competent adenoviruses. Our results as well as those of Parks et al. (1967) indicate that the latent period of the satellite virus can be shortened to 4 hr if helper adenovirus is inoculated 12 hr previously. Therefore, it is possible that after completion of a single cycle of satellite growth resulting from co-infection with satellite and adenovirus, the second satellite growth cycle may be initiated as early as 14 hr after the original mixed infection. With high multiplicities the release of complete satellite takes place 16 to 20 hr after the co-infection. Clearly, for an accurate assay by immunofluorescence, material should be harvested by 22 hr after co-infection with helper adenovirus when the influence of the secondary spread of the satellite infectivity is negligible. Thus the immunofluorescence assay for the adeno-satellite virus is one of the most rapid titration methods available for viral infectivity.

Two direct methods for assay of the satellite virus have been developed, electron microscopic particle counting (Parks et al. 1967) and complement fixation with specific antiserum (Hoggan et al. 1966). These methods detect total rather than infectious satellite particles. Electron microscope particle counts were made on selected preparations of satellite virus and indicated that the ratio of particle count to fluorescent infectivity units was of the order of 30 to 100. This ratio compares reasonably well with data for other types of viruses (Sharp, 1965).

The immunofluorescence assay was used for following the production of intracellular satellite virus in tissue culture during a single cycle of growth. In co-infected cells, particle counts indicated that both satellite and helper adenovirus particles could be first detected 12 to 14 hr later (Parks et al. 1967); however, intracellular satellite antigen was detectable in such cells about 4 hr before the detection of adenovirus antigen.

The results of the present experiments which indicate that the events in the adenovirus replication cycle must proceed for at least 10 hr before the satellite virus can be synthesized, are in agreement with those obtained by particle counting (Parks et al. 1967). In a study of the enhancement of thymidine kinase activity following infection of GMK cells with a number of DNA viruses, including SV 40 and adenovirus, Kit et al (1965) showed that increase in enzyme activity started about 10 to 11 hr after infection. In addition, there is strong evidence (Dubbs & Kit, 1965; Kit & Dubbs, 1965) that the thymidine kinases induced by two other DNA viruses, vaccinia and
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herpes simplex, are newly synthesized enzymes under virus control. Some enzyme produced under the control of the adenovirus genome may possibly perform a function in the synthesis of the satellite virus. The fact that only 4 hr are needed for the production of viable satellite virus in cells suitably primed with a competent helper adenovirus indicates that the events leading to the final maturation of the satellite virus particles are relatively rapid.

This investigation was aided in part by U.S. Public Health Service grants AI 05382 and 5 TI AI 74, from the National Institute of Allergy and Infectious Diseases, and CA 04600, from the National Cancer Institute.

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(Received 24 October 1966)