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THE ISOLATION OF TYPE 5 ADENOVIRUS USING A FLUOROCARBON; COMBINED MORPHOLOGICAL AND BIOLOGICAL STUDIES

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PURIFIED suspensions of both vaccinia virus (Epstein, 1958*a*) and the Rous virus (Epstein, 1958*b*) have recently been prepared from infected chorioallantoic membranes by means of a fluorocarbon treatment based on that of Gessler, Bender and Parkinson (1956). These suspensions were shown to be almost entirely free of formed host-cell components since, when high speed centrifugation was applied to them and samples of the resulting pellets were examined in the electron microscope, only two zones were found, one composed entirely of uniform particles and the other of amorphous gelatinous material (Epstein, 1958*a* and *b*); no other structures were detected. By making biological tests at each step in the centrifugation and electron microscopy procedures, biological activity was shown to be associated with the uniform particles, thus indicating their viral nature (Epstein, 1958*a* and *b*).

Samples of the virus-containing zones of the pellets fixed with permanganate (Luft, 1956) provided ideal material for electron microscopy and permitted the fine structure of vaccinia virus (Epstein, 1958*a*) and the Rous virus (Epstein, 1958*b*) to be investigated. Additional work with the latter agent determined the site and nature of its nucleic acid (Epstein, 1958*c*; Epstein and Holt, 1958) and demonstrated the further suitability of material of this type for study by integrated electron microscopical and cytochemical procedures.

Thus, the various techniques which were used in close combination provided valuable methods for the isolation and identification of viruses and for the study of their morphology and composition. In view of the encouraging results obtained with these methods in the case of vaccinia virus (Epstein, 1958*a* and *d*; Holt and Epstein, 1958) and the Rous virus (Epstein, 1958*b* and *c*; Epstein and Holt, 1958) it was thought that they might usefully be applied to some other agent. An infective virus of classical behaviour and medium size was decided upon so that its structure and composition could be compared with the medium sized tumour-producing Rous virus already investigated.

An adenovirus was accordingly selected and was grown, for the experiments, in HeLa cell cultures in the manner described by Pereira and Kelly (1957).

Although it was known that adenovirus from tissue cultures maintained its complement-fixing titre unimpaired after two treatments with fluorocarbon but not after more, and that one such treatment removed serologically active host antigens (Hamparian, Müller and Hummeler, 1958), no information was available regarding the state of the viral antigen after the treatments, or the presence or absence of serologically inactive associated host substances. As a first step, therefore, an investigation was made into the purifying effect on adenovirus of treatment with fluorocarbon and this was linked with biological experiments designed to identify the particles isolated. The methods used were based on those developed for earlier work (Epstein, 1958*a*) and the present communication reports the results which have been obtained ; the findings of integrated electron microscopical and cytochemical studies on the structure and composition of the adenovirus are presented elsewhere (Epstein, 1959 ; Epstein, Holt and Powell, 1960).

MATERIALS AND METHODS

Virus strain.—Type 5 adenovirus of the prototype strain has been used ; it was received, through the kindness of Dr. H. G. Pereira, in pooled HeLa cells from infected cultures showing advanced cytopathic effects and has subsequently been passed in stock cultures of these cells.

Stock HeLa cell cultures.—Stock cultures of HeLa cells were grown in flat 8 oz. medical bottles (United Bottle Manufacturers Ltd., London, W.C.2) with rubber stoppers, and when confluent sheets of cells had developed, were used either as a source of fresh cultures or for the propagation of virus. The methods and media employed were essentially those described by Pereira and Kelly (1957), their Medium I being used for growing the cells and Medium III for maintaining them after infection with the virus ; the bottles, however, were incubated at 37° in a horizontal position and the balanced saline solution used throughout differed in that 0·1 g. phenol red, 0·25 g. benzylpenicillin, 0·25 g. streptomycin and 0·125 g. sodium bicarbonate were incorporated in each litre.

Suspending fluid.—The composition of the suspending fluid used for preparing the stock virus and in the titrations has been described elsewhere (Epstein, 1958*a*).

Stock virus.—Batches of stock virus were prepared from time to time during the course of the present series of experiments. For each batch, pooled infected HeLa cells from 6 to 8 stock cultures with marked cytopathic changes were treated with fluorocarbon in the manner

EXPLANATION OF PLATES

All the figures are electron micrographs of sections cut through various regions of pellets prepared from fluorocarbon-treated suspensions of adenovirus.

FIG. 1.—Survey picture of a typical area in the virus-containing portion of the dense white zone of a pellet. Uniform virus particles 60 mμ in diameter are the only formed structures present. ×20,000.

FIG. 2.—Part of the virus-containing zone in which some of the particles are apparently arranged in crystalloid array. ×25,000.

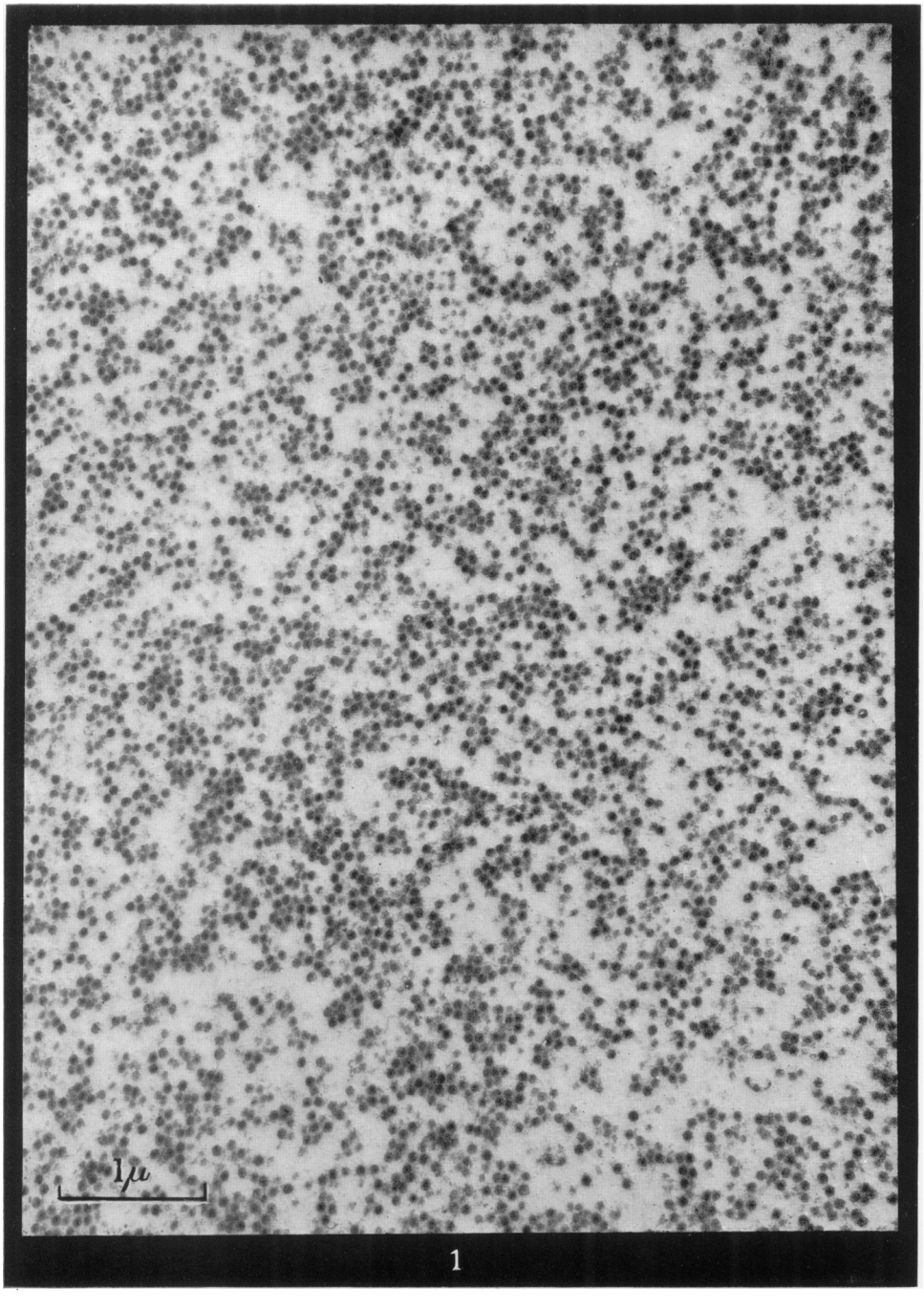
FIG. 3.—Small area of the typical virus-containing part of a pellet showing its composition in greater detail. The virus can be seen to have a hexagonal profile and to contain a central electron dense nucleoid surrounded by a less dense viroplasm. ×60,000.

FIG. 4.—Round elements from the thin basal stratum of the dense white zone ; virus particles are scattered amongst the debris. ×12,500.

FIG. 5.—Membranous structures, perhaps microsomes, from a different part of the same layer as that shown in Fig. 4. Scattered virus particles are also apparent here. ×12,500.

FIG. 6.—Survey picture of a typical part of the gelatinous region of a pellet. ×10,000.

FIG. 7.—Detail of a small area of the gelatinous zone of a pellet. Beaded threads can be readily seen. ×80,000.



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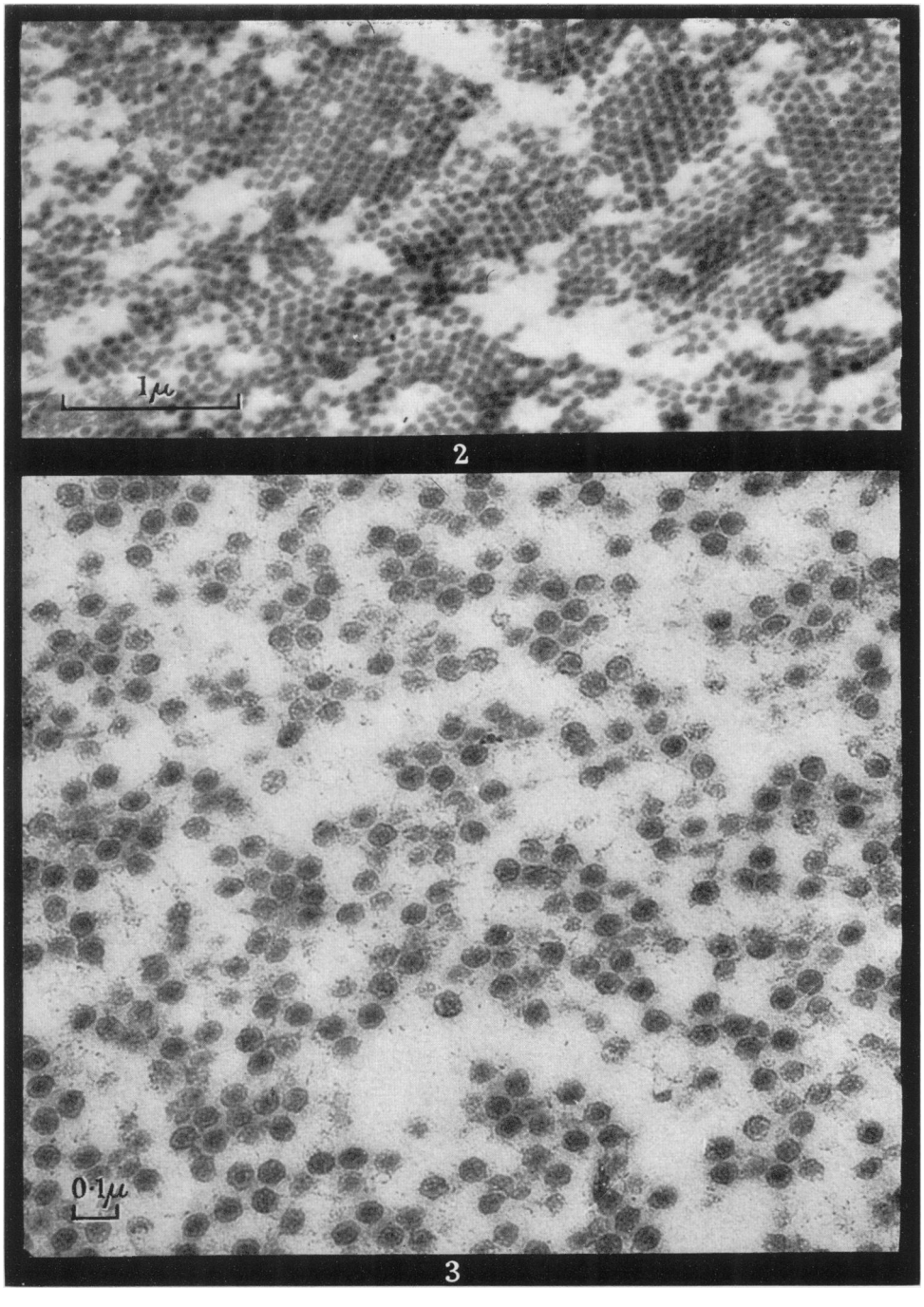
Epstein and Powell.



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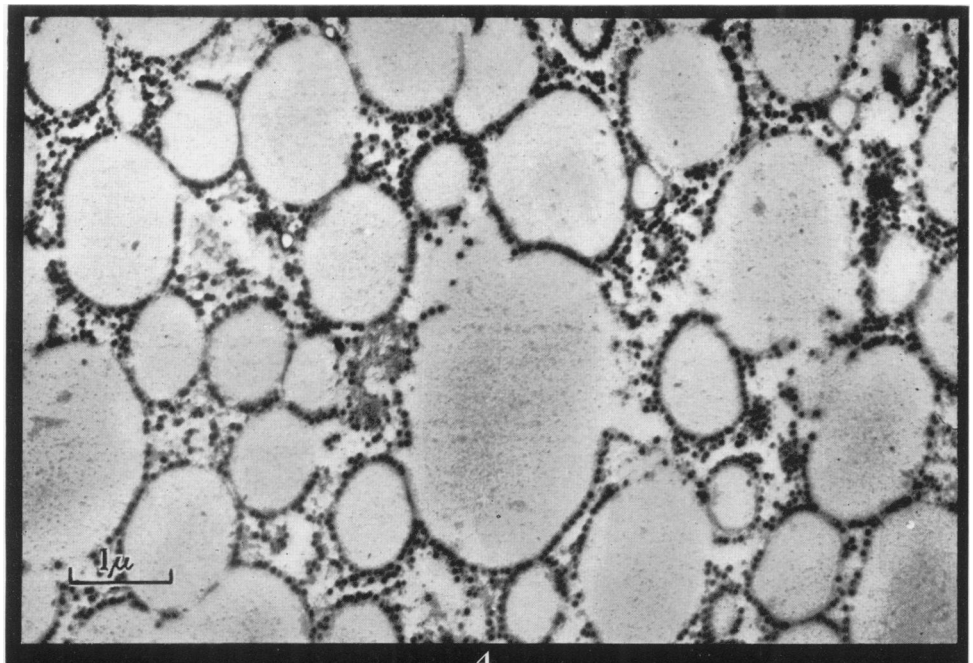

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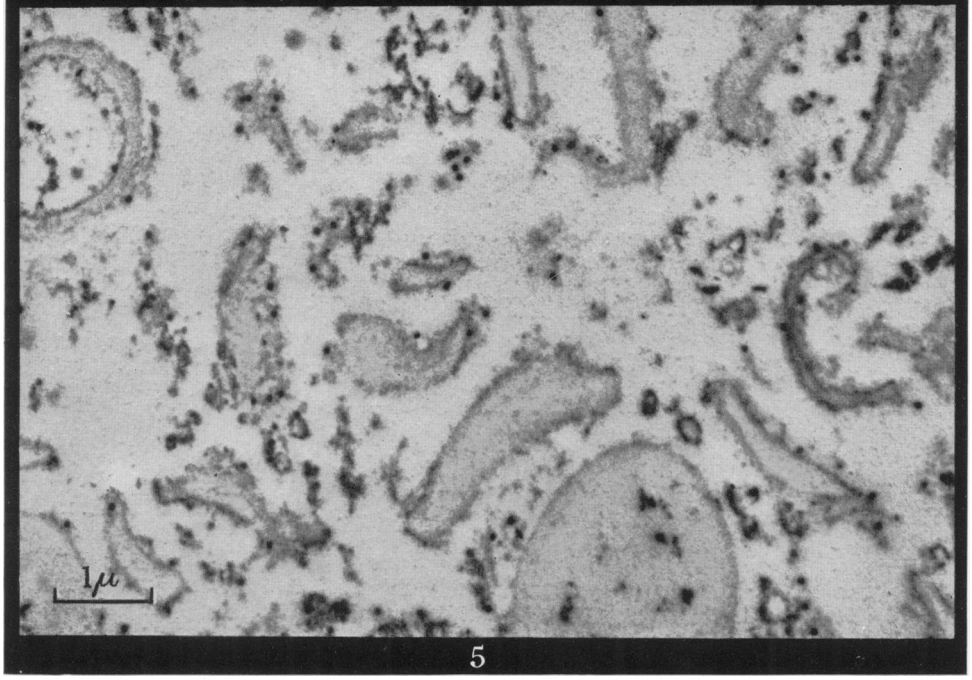

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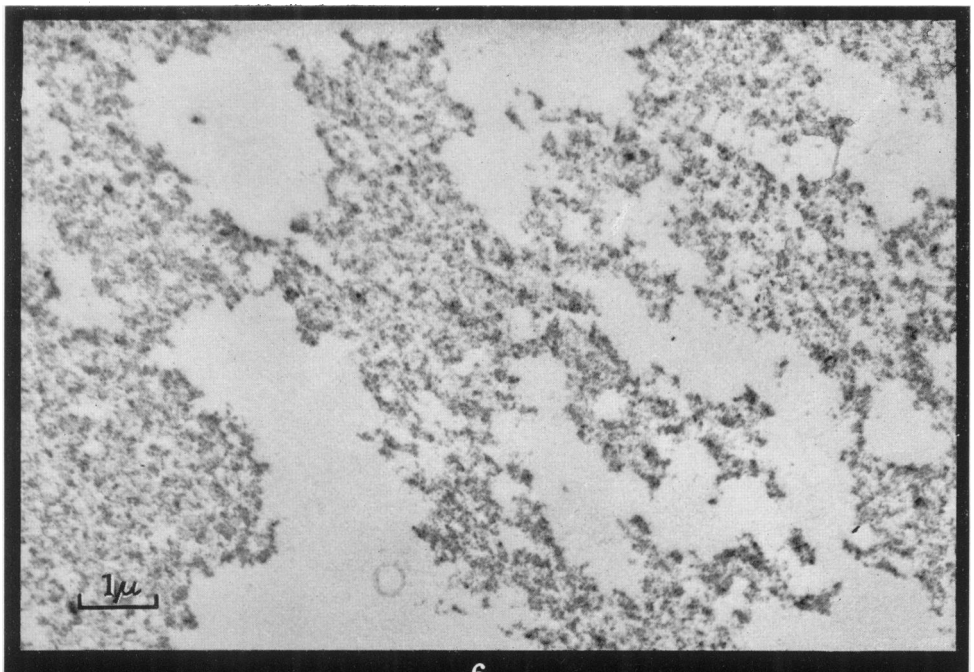

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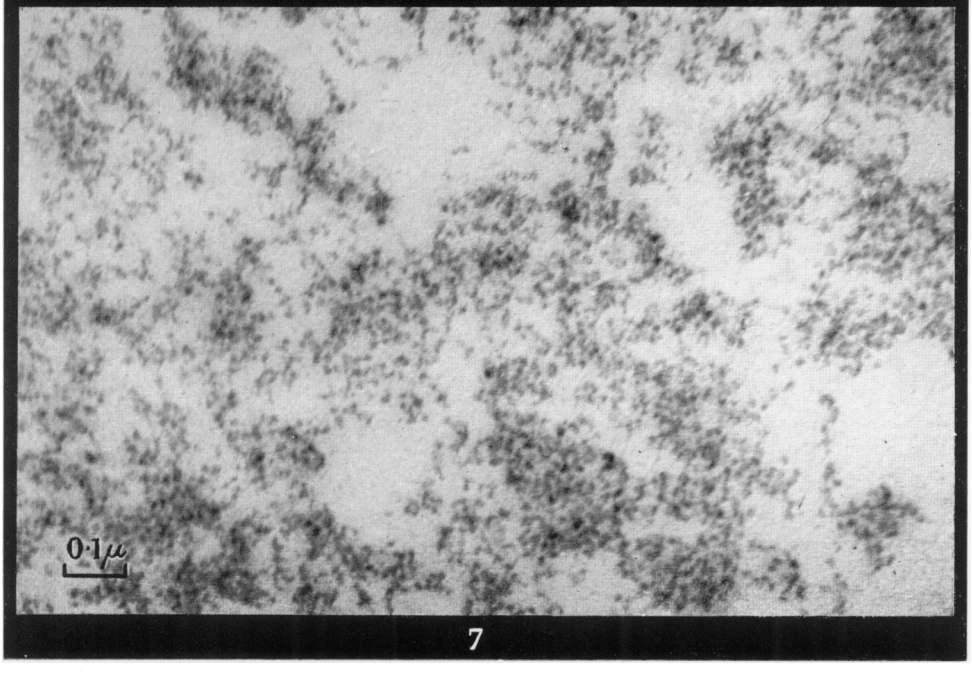

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described below. The virus-containing watery layer from either the first or the third treatment was mixed with suspending fluid, it being found convenient usually to use equal volumes. The resulting mixture was taken as the stock virus and stored in sealed hard glass ampoules in volumes of about 1 ml. at −70° until required.

Preparation of fluorocarbon-treated suspensions.

For each experiment the thawed contents of an ampoule of stock virus were added to 100 ml. of Medium III (Pereira and Kelly, 1957) which was then divided between 6 to 8 stock HeLa cultures containing confluent sheets of cells. The infected cultures were incubated in a horizontal position for 3 days at 37°, after which advanced macroscopic cytopathic changes were present and detachment of some parts of the cell sheets was usually beginning. Slight shaking of the bottles detached the remainder of the cells which were then harvested by centrifuging the pooled whole cultures at 300 *g* for 2 min. and decanting the supernatant culture fluid from above the pellets of lightly packed cells.

These pellets of cells were treated with 3 applications of a fluorocarbon as previously described (Epstein, 1958*d*). The fluorocarbon used was Arcton 113 (CF_2Cl-CCl_2F) of Imperial Chemical Industries, Ltd., London, who, until recently, designated this same compound as Arcton 63; the aqueous layer from the third application of Arcton constituted the fluorocarbon-treated suspension.

Preparation of pellets for electron microscopy from fluorocarbon-treated suspensions.

The suspensions were subjected to high speed centrifugation and samples were taken from the resulting pellets for permanganate fixation (Luft, 1956), dehydration, embedding and sectioning for electron microscopy, as previously described (Epstein, 1958*a*).

Methods used in assay of virus

Taking of samples.—For bio-assay, samples of fluids and pellets were taken and handled before inoculation as previously described (Epstein, 1958*a*).

Inoculations into HeLa cell tube cultures.—Inoculations were made into tube cultures of HeLa cells prepared 24 hr. in advance by the techniques of Pereira and Kelly (1957) in their fluid Medium II. The inoculum consisted of 0·1 ml. and 4 tubes were used for each dilution of a sample. After inoculation, rabbit serum in balanced saline was added to each tube (Pereira and Kelly, 1957) so that the final contents were equivalent to Medium III, and the tubes were then incubated at 37° and inclined in racks at 5° to the horizontal for 28 days. During this time the medium was changed on the 7th, 14th and 21st days and the tubes were examined every 3 or 4 days for cytopathic changes whose presence or absence was recorded.

General considerations

The various precautions and technical points discussed previously (Epstein, 1958*a*) were taken into account for the present work.

The biological parts of the experiments were done with strict aseptic technique; tests for the presence of contaminating bacteria were negative.

For the electron microscopy, a Philips electron microscope (EM 100) was used with a high resolution objective lens, 30μ objective aperture and flat Wehnelt cylinder. Sections were mounted on carbon coated copper grids (Watson, 1956) and were examined without removal of the embedding medium; electron micrographs were taken using an accelerating voltage of 60 kv.

Experimental procedure

Experiments 1 *and* 2.—In each of these experiments a pellet was made from a fluorocarbon-treated suspension; samples were then taken from all areas of the pellet and were prepared for electron microscopy. The samples were investigated with the electron microscope by examining serial sections cut from them at various levels.

Experiments 3 *to* 7.—In these further experiments, pellets were prepared and treated as before for morphological investigation. Meanwhile samples were taken for bio-assay from the fluorocarbon-treated suspension, the supernatant fluid above the pellet (Spinco supernatant) and from the fluids in which samples cut from various regions of the pellets had been re-suspended (Epstein, 1958*a*). All these samples were diluted in serial 10-fold steps with

suspending fluid using a clean pipette for each dilution, and portions of the various dilutions were inoculated into tube cultures of HeLa cells.

Calculation of results

From the number of tubes of HeLa cells which developed cytopathic changes following the inoculation of each dilution of a sample (Table), that dilution of the sample which would have caused the changes in 50 per cent of the tubes when given in a dose of 1 ml. was calculated. The method of calculation used was that of Reed and Muench (1938) and the results have been recorded in the Table under the heading of Lesion Dilution 50 (LD50).

RESULTS

Macroscopic appearance of the pellets

The pellets measured about 4–5 mm. diameter and had an appearance which was somewhat similar to that of pellets prepared in the same manner from chorioallantoic membranes infected with vaccinia or Rous virus (Epstein, 1958*a*, *b*). With the present material, however, the ring was faint and less sharply demarcated, the tongue likewise faint, and the dense white zone abundant; it frequently exceeded 2 mm. in diameter and extended into the base of the tongue. The rest of the pellet consisted of transparent, moderately firm jelly covering and merging into the other regions, just as was the case with pellets prepared from vaccinia and Rous virus-infected material.

Electron microscopy of the pellets

Dense white zone.—The dense white zone consisted almost entirely of uniform particles measuring about 60 mμ in diameter; the particles were mostly scattered evenly at random (Fig. 1), but aggregation in arrays which were possibly crystalline was also observed in the lower layers of the zone (Fig. 2). At higher magnification the particles were seen to be hexagonal in outline and to contain a central electron dense nucleoid surrounded by a less dense outer zone or viroplasm (Fig. 3). In the extensive area occupied by the particles no other formed elements were observed (Fig. 1, 2 and 3), but below this, forming a very thin basal layer to the dense white zone, cell debris was found. It consisted in some parts of round, relatively large, elements (Fig. 4), and in other parts of membrane bounded structures (Fig. 5); 60 mμ particles were scattered in both types of debris (Fig. 4 and 5).

Gelatinous zones.—The jelly, which composed the rest of the pellet, consisted of uniform fuzzy material arranged in strands (Fig. 6). In the lower layers of jelly from the ring and tongue areas these strands showed some packing. At high magnification the jelly was observed to be made up of fine beaded threads (Fig. 7).

Combined electron microscopy and titrations

The Table shows the results of the experiments in which the biological activity of samples taken at various stages during the preparation of pellets was investigated and related to the morphological observations made with the electron microscope on samples from different regions of each pellet.

It is evident that the infectivity of each suspension was very much greater than that of the supernatant fluid (Spinco supernatant) above the pellet resulting from high speed centrifugation of the suspension (Table). After such centrifuga-

TABLE—*The Biological Activity of Samples Taken at Different Stages During the Preparation of Pellets from Fluorocarbon-treated Suspensions (Titration in Tube Cultures of HeLa Cells)*

Expt. No.	Sample	Dilutions of sample and number of tubes with cytopathic changes from 4 inoculations of each dilution										LD_{50} per 1-ml. sample
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	
3	Fluorocarbon-treated suspension	—	—	4/4	4/4	4/4	4/4	3/4	2/4	0/4	0/3*	$10^{-8.8}$
	Spinco supernatant	—	—	4/4	4/4	3/4	0/4	0/4	—	—	—	$10^{-6.3}$
	White pellet	—	—	4/4	1/4	0/4	0/4	0/4	0/4	—	—	$10^{-4.6}$
4	Fluorocarbon-treated suspension	—	—	—	4/4	4/4	2/4	0/4	0/4	0/4	0/4	10^{-7}
	White jelly	—	4/4	4/4	0/4	0/4	0/4	0/4	—	—	—	$10^{-4.5}$
	Pellet jelly	4/4	4/4	0/4	0/4	0/4	—	—	—	—	—	$10^{-3.5}$
5	Fluorocarbon-treated suspension	—	—	—	—	4/4	4/4	4/4	1/4	1/4	0/4	$10^{-8.8}$
	Spinco supernatant	—	—	4/4	4/4	4/4	0/4	0/4	—	—	—	$10^{-6.5}$
	White pellet	—	4/4	2/4	1/4	0/4	0/4	0/4	—	—	—	$10^{-4.2}$
	Pellet jelly	4/4	2/4	0/3*	0/4	0/4	—	—	—	—	—	10^{-3}
6	Fluorocarbon-treated suspension	—	—	—	4/4	4/4	4/4	3/4	1/4	0/4	0/4	$10^{-8.5}$
	Spinco supernatant	—	—	4/4	2/4	2/4	0/4	0/4	0/4	—	—	$10^{-5.5}$
	White pellet	—	3/4	2/4	0/4	0/4	0/4	0/3*	—	—	—	$10^{-3.8}$
	Pellet jelly	4/4	1/4	1/4	0/4	0/4	—	—	—	—	—	$10^{-2.8}$
7	Fluorocarbon-treated suspension	—	—	—	4/4	4/4	4/4	1/4	0/4	0/4	0/4	$10^{-7.7}$
	Spinco supernatant	—	—	3/4	1/4	1/4	0/4	0/4	0/4	—	—	$10^{-4.7}$
	White pellet	—	4/4	4/4	4/4	3/4	1/4	0/4	—	—	—	$10^{-6.5}$
	Pellet jelly	4/4	4/4	4/4	4/4	1/4	—	—	—	—	—	$10^{-5.7}$

* Culture tubes which developed bacterial contamination during incubation were discarded.

tion 99·9 per cent of the biological activity was eliminated from the suspensions on two occasions (Expt. 6 and 7) and slightly less than this on two others (Expt. 3 and 5). That this activity was deposited in the white zones of the pellets is shown by the fact that a very small sample from this zone was highly active (Expt. 3–7) even though it had been re-suspended in as great a volume of fluid (Epstein, 1958*a*) as the original suspension from which the whole pellet came; in contrast, similarly re-suspended samples from the gelatinous zones of the pellets possessed considerably less activity (Expt. 4–7).

When samples taken from the white zones of the pellets were examined in the electron microscope they were seen to consist entirely of uniform hexagonal particles about 60 mμ in diameter (Fig. 1, 2 and 3) except for the cell debris forming the very narrow basal stratum below (Fig. 4 and 5). Samples of the gelatinous areas of the pellets were found to be free of such particles (Fig. 6 and 7) when examined in this way.

DISCUSSION

Although three applications of fluorocarbon (Epstein, 1958*a*) were used in preparing suspensions for the present experiments, free virus in such suspensions was only exposed to the fluorocarbon twice in the free state, since the second treatment was in fact a re-extraction of the original cellular starting material. The procedure followed thus took into account the findings of Hamparian,

Müller and Hummeler (1958) regarding the impairment of the specific complement-fixing antigen of adenovirus by more than two treatments with fluorocarbon.

The nature of the large round elements found (Fig. 4) is not known, but the membrane bounded structures (Fig. 5) could, despite their relatively large size, be microsomes. The latter are now considered to be derived by fragmentation from the rough surfaced elements of the endoplasmic reticulum (Palade, 1958) and the unusual largeness of the structures observed here might simply be a result of the type of fragmentation produced by the present preparation procedure. The finding of apparently smooth limiting membranes around the structures (Fig. 5) is not inconsistent with their identification as microsomes, since it has been known for some time that the characteristic 15 mμ ribonucleoprotein particles which are seen, after osmium fixation, studding the outside of microsomal membranes and causing their "rough" appearane (Palade and Siekevitz, 1956*a* and *b*), are not preserved in material fixed with permanganate (Luft, 1956).

That a layer of cell debris was found in the pellets obtained from fluorocarbon-treated suspensions of HeLa cells infected with adenovirus, is in marked contrast to the absence of such debris in the material obtained previously from similarly treated vaccinia-infected chorioallantoic membranes (Epstein, 1958*a*). This difference is in all probability due to variation in the number of applications of fluorocarbon used in the two sets of experiments—namely three in the present series as against five in the work with vaccinia—and support for such an explanation is afforded by the results of earlier studies on fluorocarbon purification of the Rous virus. In these it was found that pellets derived from egg grown Rous nodules treated five times with the fluorocarbon contained membranous cell debris, whereas those prepared after seven treatments did not (Epstein, 1958*b*). Elimination of formed host cell components would thus appear to depend on the number of times fluorocarbon is applied and this number seems, in turn, to require variation both with the relative quantities of cells and reagents involved and with each particular cell system. The present findings taken together with the results of the work with the Rous virus (Epstein, 1958*b*) further suggest that membranous microsomal elements are the last cell structures to be removed in the course of repeated applications of fluorocarbon.

The presence of jelly in the pellets shows that fluorocarbon treatment failed to remove certain unformed contaminating host substances, just as was found to be the case earlier in the vaccinia and Rous virus experiments (Epstein, 1958*a* and *b*), and when examined in the electron microscope, the jelly from each system was morphologically similar.

From the results of the combined morphological and biological experiments it is evident that the fluorocarbon treatment used enabled suspensions to be made in which the only formed structures were vast numbers of uniform polyhedral particles 60 mμ in diameter and hexagonal in profile, together with a very small quantity of cellular debris. This follows from the fact that when high speed centrifugation was applied to the suspensions, the resulting pellets consisted only of particles (Fig. 1, 2 and 3), amorphous fuzzy material (Fig. 6) and the thin layer of cell fragments at the base of the dense white zone (Fig. 4 and 5).

The identity of the particles is indicated by the results of the titration experiments (Table). These show that when the suspensions were centrifuged their infectivity came to lie in that region of each pellet occupied only by particles and

a thin layer of cell fragments. Now, similar cell fragments without infectivity have been isolated from uninfected HeLa cells by the same techniques as those used here, whilst particles have not (Holt, Epstein and Powell, unpublished) and work reported by Pereira and Valentine (1958) has clearly shown that infection with adenovirus depends on the presence of particles, even as few as 10 being capable of forming an infective dose. The particles in question were of the same order of size as those observed here in the infective white zones of pellets, and indeed the correspondence is very close if allowance is made for flattening during the air-drying of the preparation method which Pereira and Valentine (1958) used. Thus, the uniform 60 mμ particles which formed the bulk of the white zones appeared similar to the adenovirus in many respects and were associated with a similar type of infectivity.

A further reason for concluding that the particles were in fact the adenovirus is provided by the fact that such particles have been observed with the electron microscope in adeno-infected tissue culture cells by many workers (Kjellén, Lagermalm, Svedmyr and Thorsson, 1955 ; Morgan, Howe, Rose and Moore, 1956 ; Harford, Hamlin, Parker and van Ravenswaay, 1956), both scattered in the nuclei and also frequently arranged there in striking, apparently crystalline, arrays, very similar to those found in the pellets (Fig. 3). Although these virus crystalloids in the pellets might have arisen after extraction and purification of the particles, it is more likely that they represent intact portions of crystals present in the nuclei of the original infected HeLa cells used as starting material for each of the present experiments.

The conclusion that infectivity was associated with the particles is also supported by the similar results obtained in earlier experiments made with vaccinia and the Rous virus using the same technical approach (Epstein, 1958*a* and *b*) ; because of the uniform findings the validity of any one set of experiments is enhanced by the others.

In this connection it should be noticed that the difference in infectivity between samples of the gelatinous and white zones of adeno pellets was much the same as that found in Rous pellets (Epstein, 1958*b*), and much less than that in vaccinia pellets (Epstein, 1958*a*). The difficulty of avoiding clumping and aggregation when samples of the relatively small Rous virus were re-suspended for titrating has already been pointed out (Epstein, 1958*b*) and it would seem that the same difficulty has now been encountered with the similarly sized adenovirus.

Besides indicating the viral nature of the particles from adenovirus-infected tissue cultures, the present work shows that, by the techniques used, these particles can be obtained entirely free from formed host elements in a circumscribed area of pellet (Fig. 1). When isolated in this way the particles have still been morphologically intact (Fig. 1, 2 and 3) and associated with infectivity (Table) and it seems probable that they could be freed from the other components in the pellets by density gradient centrifugation, or adsorption on and elution from, appropriate separating columns.

However, even without any further treatment, the purified particles in the white zones of pellets provide admirable material for the study of virus structure and composition. This has already been demonstrated in the case of vaccinia (Epstein, 1958*a*) and the Rous virus (Epstein, 1958*b* and *c* ; Epstein and Holt, 1958) and recent investigations reported elsewhere (Epstein, Holt and Powell, 1960) now add confirmation with adenovirus.

SUMMARY

An investigation into the purifying effect of a fluorocarbon (CF_2Cl-CCl_2F) on adenovirus grown in HeLa cell cultures is described. Pellets made by high speed centrifugation of treated virus suspensions have been prepared for sectioning for electron microscopy so that their purity could be assessed by direct observation ; this morphological work was combined with tests for biological activity.

The results show that the fluorocarbon treatment yielded suspensions which contained amorphous host substances, a small amount of formed cellular debris and very large quantities of uniform 60 mμ particles ; the latter were shown in the biological tests to be associated with infectivity, thus indicating their viral nature.

Both the reasons for concluding that the cell debris is unassociated with infectivity and the significance of this material in relation to fluorocarbon purification are discussed.

The usefulness of the extensive zone in the pellets in which purified virus was found isolated from all formed host debris is also considered.

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