AN INVESTIGATION INTO THE PURIFYING EFFECT OF A FLUOROCARBON ON VACCINIA VIRUS

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Two main problems have been largely responsible for hampering work on the chemical composition of animal viruses.

Firstly, there is the difficulty of separating the virus particles from host cell constituents liberated along with them at the time of cell disruption. Although some animal viruses, such as influenza or vaccinia, have properties which make them relatively easier to purify than others (Ada and Perry, 1954 ; Craigie, 1932 ; Hoagland, Smadel and Rivers, 1940), members of the animal virus group as a whole are infinitely harder to obtain pure than, for example, certain insect viruses (Smith and Williams, 1958) and plant viruses (Steere, 1956).

The second problem is an extension of the first ; it consists, as has been pointed out elsewhere (Burnet, 1955), in the difficulty of establishing a criterion of purity once preparations of virus particles have been obtained. As Burnet (1955) has stressed, it is the absence of an adequate criterion on this crucial point which has rendered suspect so many investigations on the composition of animal viruses.

With regard to the first of these two problems, it has been claimed by Gessler, Bender and Parkinson (1956) that viruses can be purified and separated from host cell constituents if extracted from infected tissue by homogenization in a water-fluorocarbon mixture. The work reported did not include any adequate biological, morphological or other tests for the purity of the virus preparations which were made, but, as has been mentioned above, the difficulties in the way of applying satisfactory checks of purity form a major obstacle in this field. However, it has been shown quite recently in complement fixation experiments (Hummeler and Hamparian, 1957 ; Hamparian, Müller and Hummeler, 1958) that treatment of various animal virus suspensions with fluorocarbon did in fact effectively remove host antigens and anti-complementary activity, so that fluorocarbon might indeed be of value for the purification of such viruses.

Concerning criteria for assessing the purity of virus material, a new approach is suggested by some recent investigations of Palade and Siekevitz (1956*a* and 1956*b*). These workers have shown in an elegant series of studies that ultra-thin sectioning techniques for electron microscopy can be applied to pellets of cell fractions obtained by differential centrifugation. This provides a means of judging the homogeneity and the purity, morphologically, of the pellets down to the level of fine structure which can be resolved with the electron misroscope. Although such a check cannot yield information regarding the adsorption or loss of soluble substances by structures visible in a pellet, at least direct observations

on the morphological homogeneity of these structures provide a criterion of purity far superior to any hitherto available and one which can be applied to virus pellets just as readily as to those composed of cell fractions.

In view of the success obtained by Hummeler and Hamparian (1957) with fluorocarbon in their serological work, it was considered that an investigation of the virus purifying effect of a compound of this type would be valuable. Vaccinia virus was selected for this since it had been used in the original experiments with fluorocarbon (Gesslers *et al.*, 1956) and had the advantage of being relatively large and relatively easy to handle.

Preparations of the virus which had been treated with fluorocarbon have been subjected to high speed centrifugation in order to obtain pellets of the formed elements present. The pellets were fixed, embedded and sectioned for electron microscopy so that their purity might be checked by direct observation in the way introduced by Palade and Siekevitz (1956*a* and 1956*b*). Permanganate fixation (Luft, 1956) has been used throughout since preliminary investigations had shown that this revealed much unsuspected fine structure in the mature virus particles (Epstein, 1958*a*) and, in marked contrast to osmium-sucrose fixation (Palade, 1952 ; Caulfield, 1957), gave excellent preservation right through all samples of the pellets. In combination with the morphological work, checks have been made on the biological activity of the virus materials at each stage.

The present communication reports the results which have been obtained ; the findings of parallel cytochemical studies on the pellets of fluorocarbon-purified vaccinia virus are presented elsewhere (Holt and Epstein, 1958).

MATERIALS AND METHODS

Virus strain.—A Lister Institute strain of vaccinia virus was used ; it was obtained in 1954, through the kindness of Dr. D. McClean, as pulp from the 8th mouse skin passage. The virus was subsequently passed 11 times in the cells of mouse ascites Sarcoma 37 *in vivo* and 6 times on the chorio-allantoic membrane of chick embryos ; between some of the passages the virus was stored at −70° in sealed ampoules.

Eggs.—Fertile chickens' eggs were obtained throughout from the same source ; they were used after the embryos had been incubated for 10 to 11 days at 37°.

Suspending fluid.—The suspending fluid used for preparing the stock virus pool and in the titrations, consisted of 5 per cent inactivated rabbit serum in 0·01 M phosphate buffer at pH 7·38, brought to isotonicity by the addition of NaCl (0·792 g. per 100 ml.). The serum was inactivated by heating it to 56° for 30 min. shortly before use.

Stock virus pool.—Stock virus was prepared by grinding 5 infected chorio-allantoic membranes with sand and placing the ground-up material in 10 ml. of suspending fluid. The suspension was centrifuged at 300 *g* for 3 min. and the resulting supernatant fluid was kept as the virus pool ; it was stored in sealed hard glass ampoules in 1 ml. volumes at −70° until required.

Preparation of fluorocarbon-treated virus suspensions

For each experiment, the thawed contents of an ampoule of stock virus were diluted in serial ten-fold steps with suspending fluid and 0·1 ml. samples of the 10^{-4} dilution were inoculated on to the chorio-allantoic membranes of a group of eggs using false air sacs prepared by the standard technique (Beveridge and Burnet, 1946) ; preliminary titrations of the stock virus pool had shown that this dilution gave maximal confluent lesions on the chorio-allantois after incubating the inoculated embryos for 72 hr. at 37°.

About 5 chorio-allantoic membranes with maximal confluent lesions following inoculation and incubation in the manner just described, were harvested. The membranes, which weighed about 3 g., were placed with 15 ml. of Earle's balanced saline solution (Earle, 1943)

and 7·5 ml. of Arcton 63 (CF_2Cl-CCl_2F of Imperial Chemical Industries Ltd., London) in the 100 ml. vortex beaker of a Nelco homogenizer (Type N.I.R.D. supplied by Measuring and Scientific Instruments Ltd., London). The homogenizer was run at top speed (14,000 r.p.m.) for 75 sec. and the homogenate was then decanted and centrifuged at 300 *g* for 1 min. causing the Arcton layer to separate out at the bottom with the aqueous layer above, sometimes topped by a thin covering of frothy scum ; this method of homogenization and centrifuging was followed throughout the preparation procedure. The aqueous layer was pipetted off and kept, whilst the Arcton layer was homogenized again with 10 ml. fresh Earle's solution and 5 ml. fresh Arcton ; after centrifugation the aqueous layer from this second treatment was pipetted off and kept. A third cycle of homogenization and centrifugation was next done on the pooled aqueous layers from the first and second runs mixed with 10 ml. fresh Arcton. This treatment was repeated a fourth and fifth time, the aqueous layer from each run being homogenized with 10 ml. fresh Arcton in the next run. After the fifth treatment the Arcton layer was dead white in colour and the aqueous layer was clear, except for a slight milky opalescence ; this final aqueous layer constituted the Arcton-treated virus suspension.

Preparation of pellets for electron microscopy from virus suspensions

About 10 ml. of virus suspension was centrifuged in the No. 40 (angle) rotor of a Spinco Model L preparative ultra-centrifuge (of Beckman Instruments Inc., Palo Alto, California) at an average of 105,000 *g* for 1 hr., the centrifuge being brought to a halt by means of the brake.

Fixation.—The supernatant was then decanted from the centrifuge tube, the tube was cut in half down its long axis and the pellet it contained was cut up in the open tube into sample cubes measuring up to 0·5 mm.; this sampling was done under a binocular dissecting microscope (Lomag of R. and J. Beck, Ltd., London, magnifying × 6·6) using two Hudson-type lachrymal sac knives. The samples were immediately transferred with one of the knives into a fixing bottle containing iced 0·6 per cent $KMnO_4$ buffered at pH 7·6 (Luft, 1956) and fixation was continued at 2° for about 1 hr.

Dehydration and embedding.—When fixation was completed the fixative was replaced by iced 30 per cent alcohol which was kept at 2° for 15 min. and then by a further change of iced 30 per cent alcohol which was allowed to warm to room temperature during the next 15 min., as recommended by Luft (1956). The samples of pellet were further treated by passage through 50 per cent alcohol, 70 per cent alcohol, 90 per cent alcohol, three changes of 100 per cent alcohol, a mixture of equal parts of absolute alcohol and catalysed *n*-butyl methacrylate and then 3 changes of catalysed *n*-butyl methacrylate ; each change lasted 25 min. The methacrylate was catalysed by the addition of 1 per cent by weight of benzoyl peroxide. For embedding, the samples were placed in No. 00 gelatin capsules containing catalysed *n*-butyl methacrylate which had been prepolymerized for about 1 hr. at 57°. Polymerization was carried out at 58° for 14–18 hr.

Microtomy.—Sections were cut with a diamond knife (Fernández-Morán, 1956)—kindly presented to us by Professor Humberto Fernández-Morán—fitted with a holder (Epstein, 1958*b*) which made it suitable for use in a microtome designed for glass knives (Porter and Blum, 1953). The sections were flattened on 20 per cent aqueous acetone by the method of Sotelo (1957) ; those showing golden interference colours were selected for surveying the general arrangement of the pellets, whilst those showing silver interference colours were used to study the fine structure of the individual objects present. For examination, the sections were mounted on formvar coated copper grids without removal of the embedding medium.

Methods used in assay of virus

For the assay of virus in a given material, the following procedures were used :

Taking of samples.—Samples of fluids were taken with tuberculin syringes and were kept at 4°. Samples of the pellets were taken in the way described above for those used for electron microscopy ; the samples were transferred with a lachrymal sac knife to a mortar and were vigorously ground with a pestle in a few drops of suspending fluid. After the grinding, the volume of the suspending fluid was made up to 10 ml. and a portion of this was taken in a syringe and kept at 4°. Each sample in an experiment was kept stored only until all other samples were ready for use.

Inoculations.—Inoculations were made on to the chorio-allantoic membranes of eggs using false air sacs made by a method similar to that described by Scott (1948). The inoculum consisted of 0·1 ml. and either 4 eggs (Expt. 4) or 5 eggs (Expt. 5 and 6) were used for each dilution of a sample. After inoculation, the eggs were incubated for 72 hr. at 37° and were then opened ; the inoculated membranes of all live embryos were removed, floated in saline in Petri dishes and the presence or absence of pocks was recorded.

General considerations

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

For the Arcton treatment of the virus suspensions the apparatus and fluids were cooled before use to about 2° and homogenization was carried out with the vortex beakers surrounded by a bath of melting ice.

During the cycles of homogenization, clean chilled beakers were brought into use for the first, fourth and fifth runs so that the clinging froth of one run was not carried over on the beaker wall to the next, after the homogenate had been decanted for centrifugation.With the particular quantities of the fluids used it was important to adjust the position of each vortex beaker so as to leave the least possible clearance space between its base and the homogenizer blades.

For the preparation of pellets from the virus suspensions the Spinco centrifuge rotor was cooled before use to about 2° and the centrifuge refrigerator was switched on when centrifugation was started. This ensured that the material in the centrifuge did not warm up beyond 4° during the run.

Where titrations were performed, the inoculations were completed about 2½ hr. after harvesting the chorio-allantoic membranes from which the samples under test had been prepared. The different samples and their dilutions were dealt with in quick succession and material of a type which preliminary experiments had shown to be likely to have little biological activity (*e.g.*, supernatant fluid above a pellet) was inoculated before that likely to have greater activity (*e.g.*, virus suspension).

Electron microscopy was done with a Philips EM-100 fitted with a high resolution objective lens ; maximum image contrast was obtained by the use of a 30 μ objective aperture and a flat Wehnelt cylinder. Electron micrographs were taken using an accelerating voltage of 60 kV.

Experimental procedure

Experiments 1 to 3.—In each of these experiments a pellet was made from a fluorocarbon treated virus 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 sets of serial sections cut from them at various levels.

Experiments 4 to 6.—In these further experiments, pellets were prepared and treated as before ; in addition, during this procedure samples were taken from the virus suspension, the supernatant fluid above the pellet (Spinco Supernatant) and from the fluids containing re-suspended fragments cut from different regions of the pellets. These samples were diluted in serial ten-fold steps with suspending fluid and portions of the various Dilutions were used for egg inoculation.

Calculation of results

From the number of chorio-allantoic membranes which developed pocks following the inoculation of each dilution of a sample (table), that dilution of the sample which would have caused lesions in 50 per cent of chorio-allantoic membranes 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 (LD_{50}).

RESULTS

Macroscopic appearance of the pellets

The pellets measured about 4 mm. in diameter and had a characteristic appearance (Fig. 1). This consisted of a firm, dense white, round zone 1 to 2

mm. across, which lay in the upper central part of a pale grey ring; the ring occupied most of the outer portion of the pellet and there was a greyish tongue protruding into its centre from the lower edge of the white zone (Fig. 1). These regions merged into, and were covered by, transparent, moderately firm, gelatinous material which formed the upper layers of the pellet.

Electron microscopy of the pellets

Dense white zone.—The dense white zone consisted entirely of uniform particles measuring about 300 mμ by 200 mμ (Fig. 2); no other formed elements were

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EXPLANATION OF PLATES

All the figures, apart from the first one, are electron micrographs of various regions of pellets prepared from fluorocarbon-treated vaccinia virus suspensipns.

FIG. 1.—View of the lower part of a centrifuge tube showing a pellet prepared by subjecting a fluorocarbon-treated vaccinia virus suspension to an average force of 105,000 *g* for 1 hr. The pellet consists of a dense white zone lying in the upper central part of a pale grey ring. The ring occupies the outer portion of the pellet and there is a greyish tongue protruding into its centre from the lower edge of the white zone. Light reflections can be seen at the base of the tube and on the right of the pellet. × 6.

FIG. 2.—Survey picture of a section cut through a representative region of the dense white zone of a typical pellet. Randomly orientated uniform particles measuring about 300 mμ by 200 mμ are the only formed structures present. × 13,000.

FIG. 3.—Small area of a section cut through the dense white zone of a pellet showing its composition in greater detail. Varying amounts of the individual randomly orientated particles are present in the section; those which have been cut tangentially have only a thin surface sliver included, in contrast to the more substantial segment taken from those cut nearer their centres. × 40,000.

FIG. 4 to 7.—Individual mature vaccinia virus particles from sections of the dense white zones of various pellets; the particles have been cut in different planes. × 200,000.

FIG. 4.—Longitudinal section across the shortest dimension of a particle showing the slight flattening. The outer limiting membrane consists of two electron-dense layers 5 mμ apart separated by a less dense layer in between. This limiting membrane covers a narrow zone of slight electron-density (*oz*), except at the centre of each of the two widest surfaces of the particle where an electron-dense lateral body (*db*) is interposed. The narrow zone of slight electron-density is limited centrally by another membrane which in turn surrounds an inner body or nucleoid. The nucleoid itself is differentiated into a central electron-dense area with an area of less density around it.

FIG. 5.—Longitudinal section across the widest dimension of a particle; this is at right angles to the plane of the section shown in Fig. 4, and along the line A–B. The layers of the outer limiting membranes are well seen as is the discoidal shape of the central electron-dense area of the nucleoid.

FIG. 6.—Transverse slightly oblique section through a particle along the line C–D of Fig. 4. The electron-dense lateral bodies (*db*) can be seen under the limiting membrane, separated from the nucleoidal membrane by the narrow zone of slight electron-density.

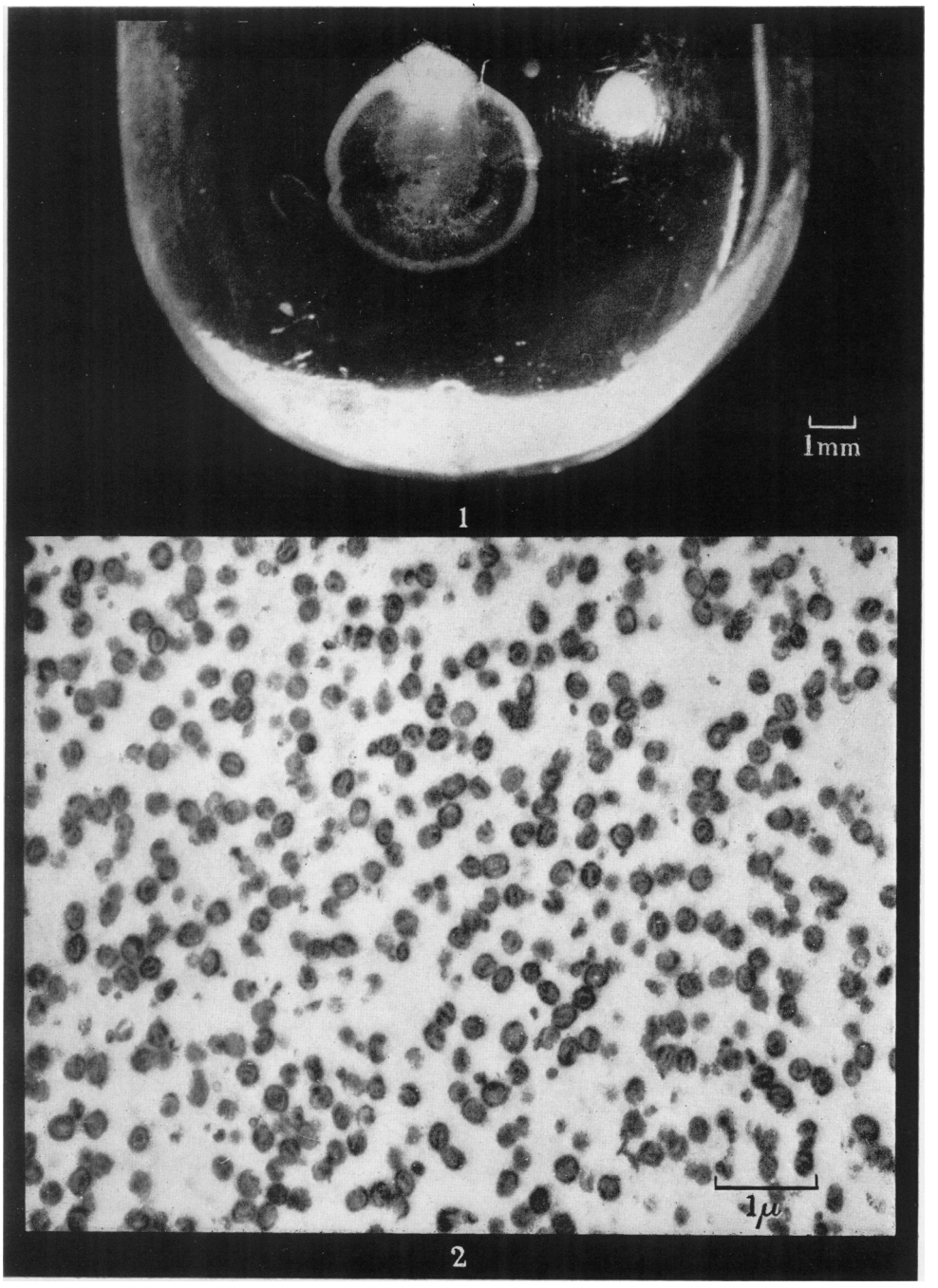
FIG. 7.—Section through a particle in almost the same plane as in Fig. 4; there is some asymmetry present and only one lateral body (*db*) has been cut through. The three-layered structure of the nucleoidal membrane is particularly clear.

FIG. 8.—Section through the gelatinous region of a pellet. × 40,000.

FIG. 9.—Detail of a small area of a section through the gelatinous region of a pellet. The granular appearance can be seen to be due to the presence of fine beaded threads. × 100,000.

FIG. 10.—Section through the area of a pellet where the dense white zone ends and the overlying jelly begins. The particles of the dense white zone are present on the right of the field with the sharply demarcated jelly covering this zone, on the left. × 30,000.

FIG. 11.—Survey picture of a section through the ring region of a pellet. The fuzzy jelly is arranged in strands forming a coarse meshwork. These are packed down into dense laminae in the lower layers of the pellet shown at the bottom of the field. × 6,000.



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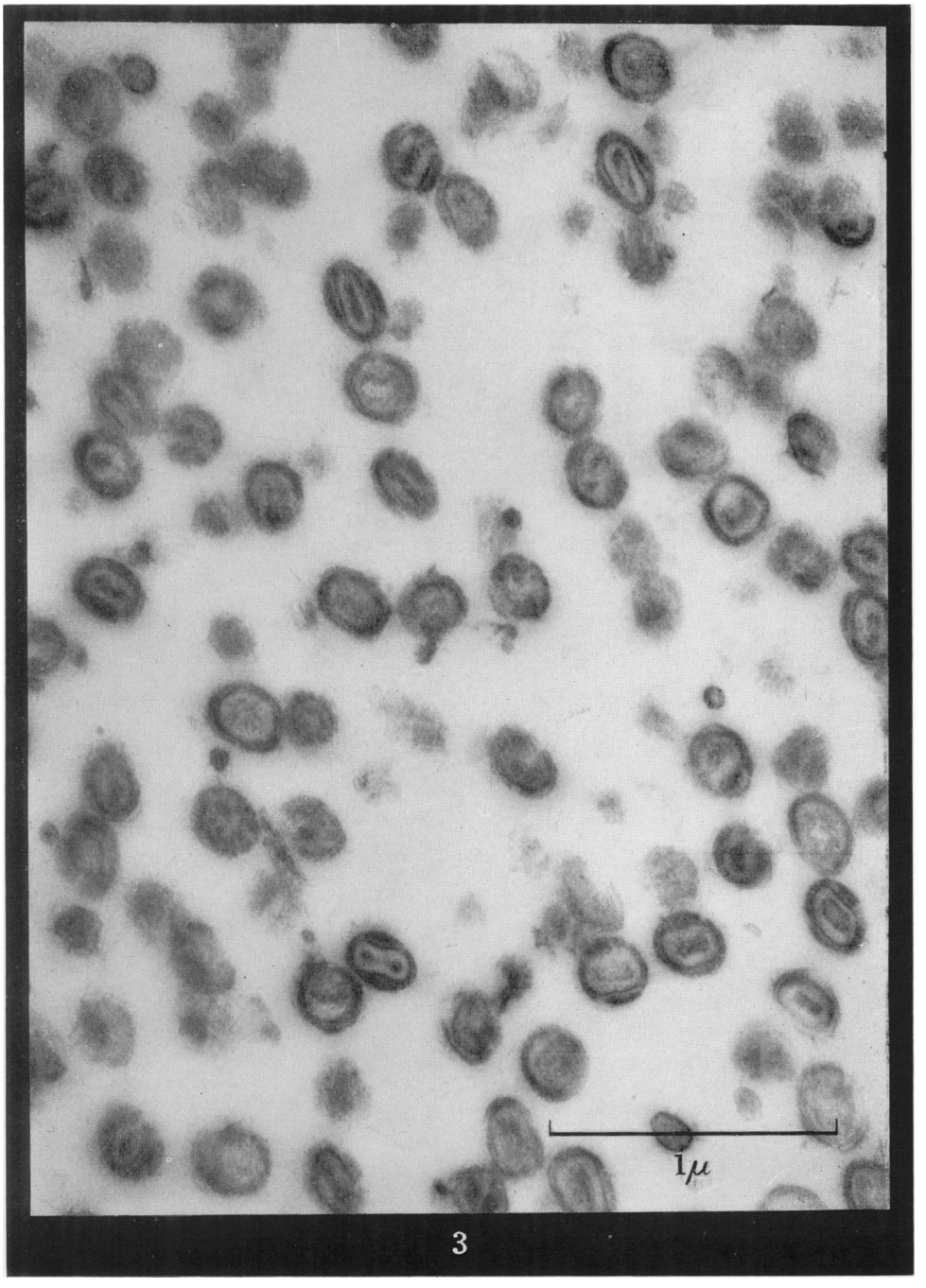

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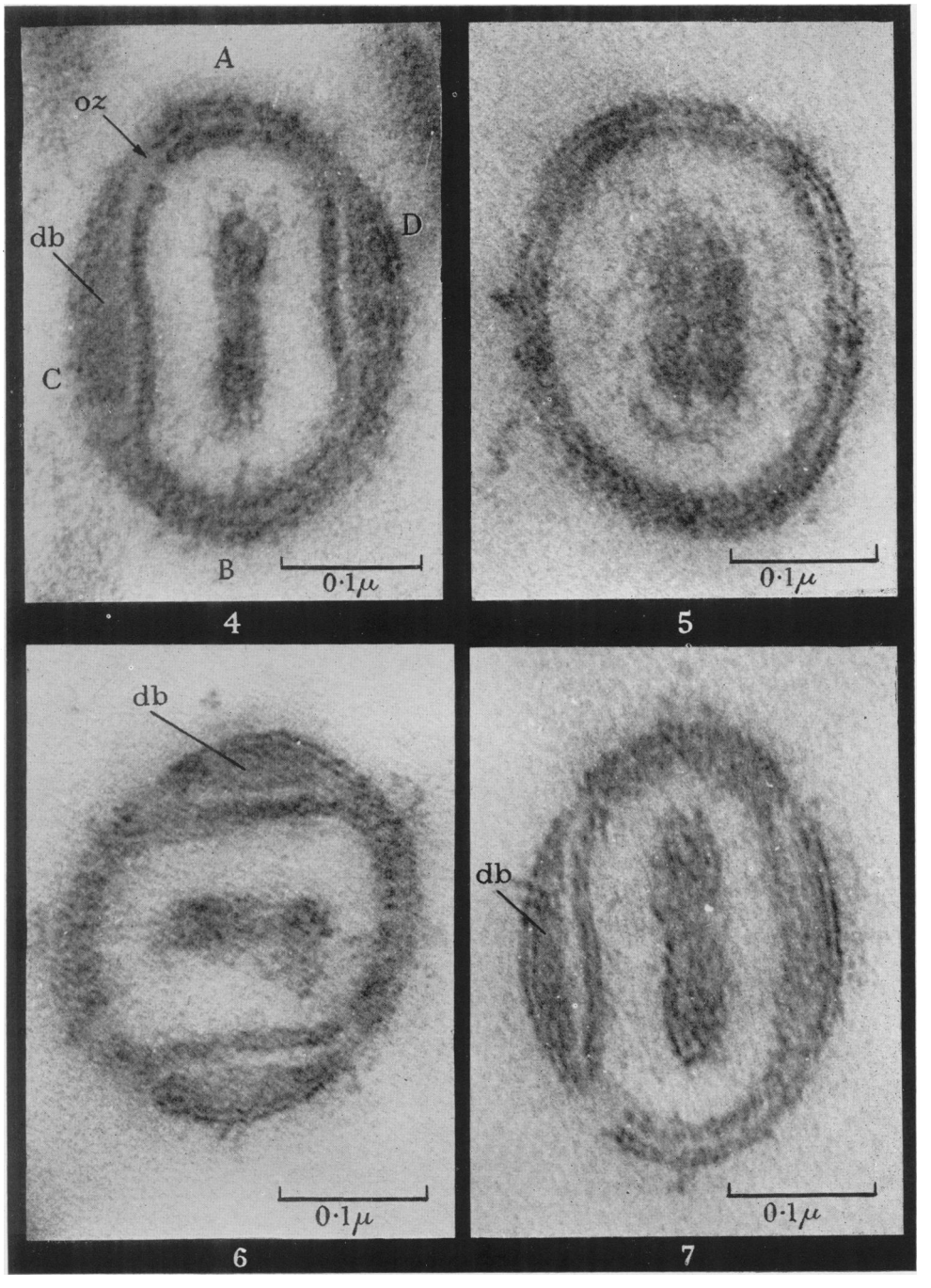

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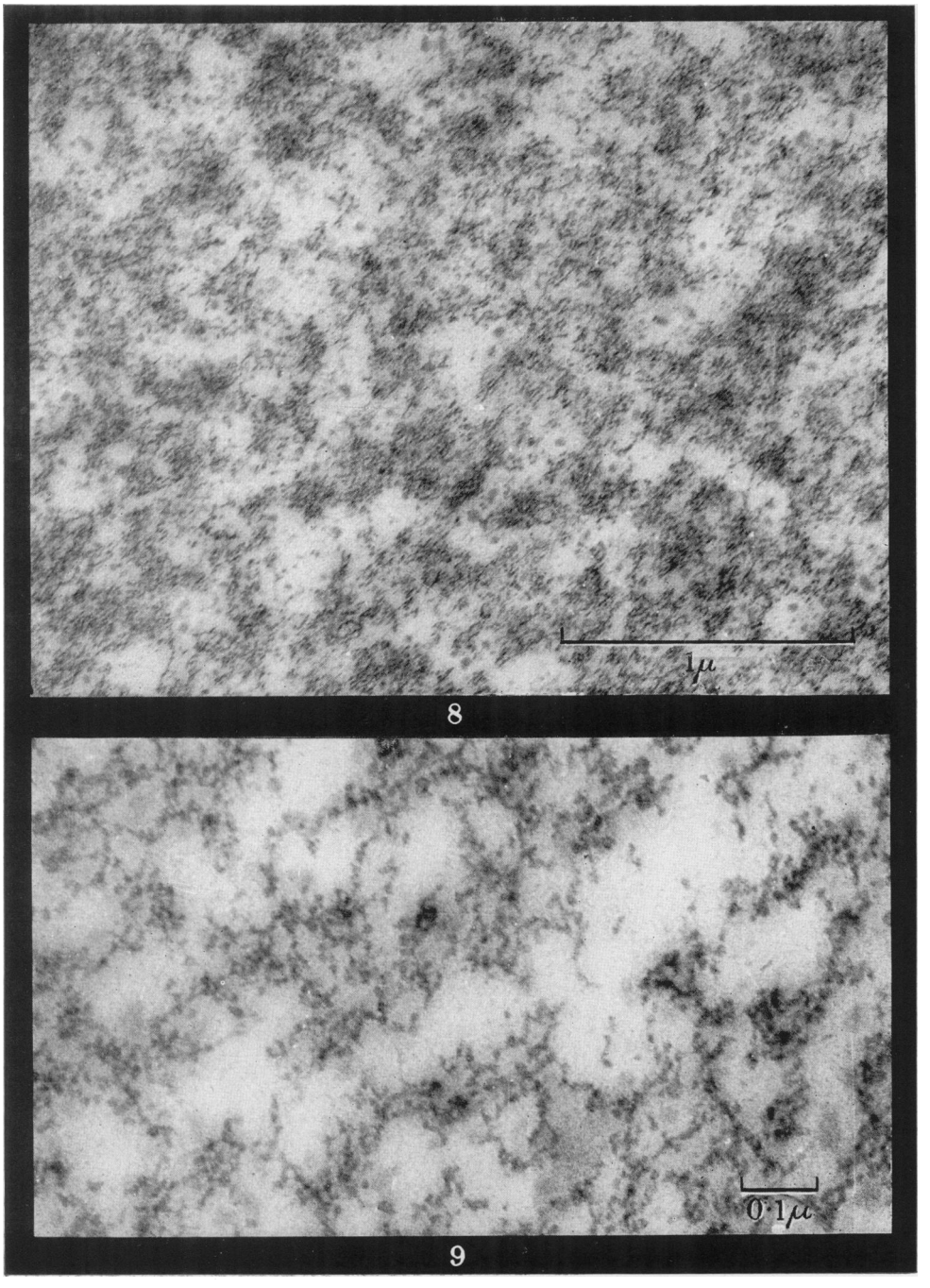

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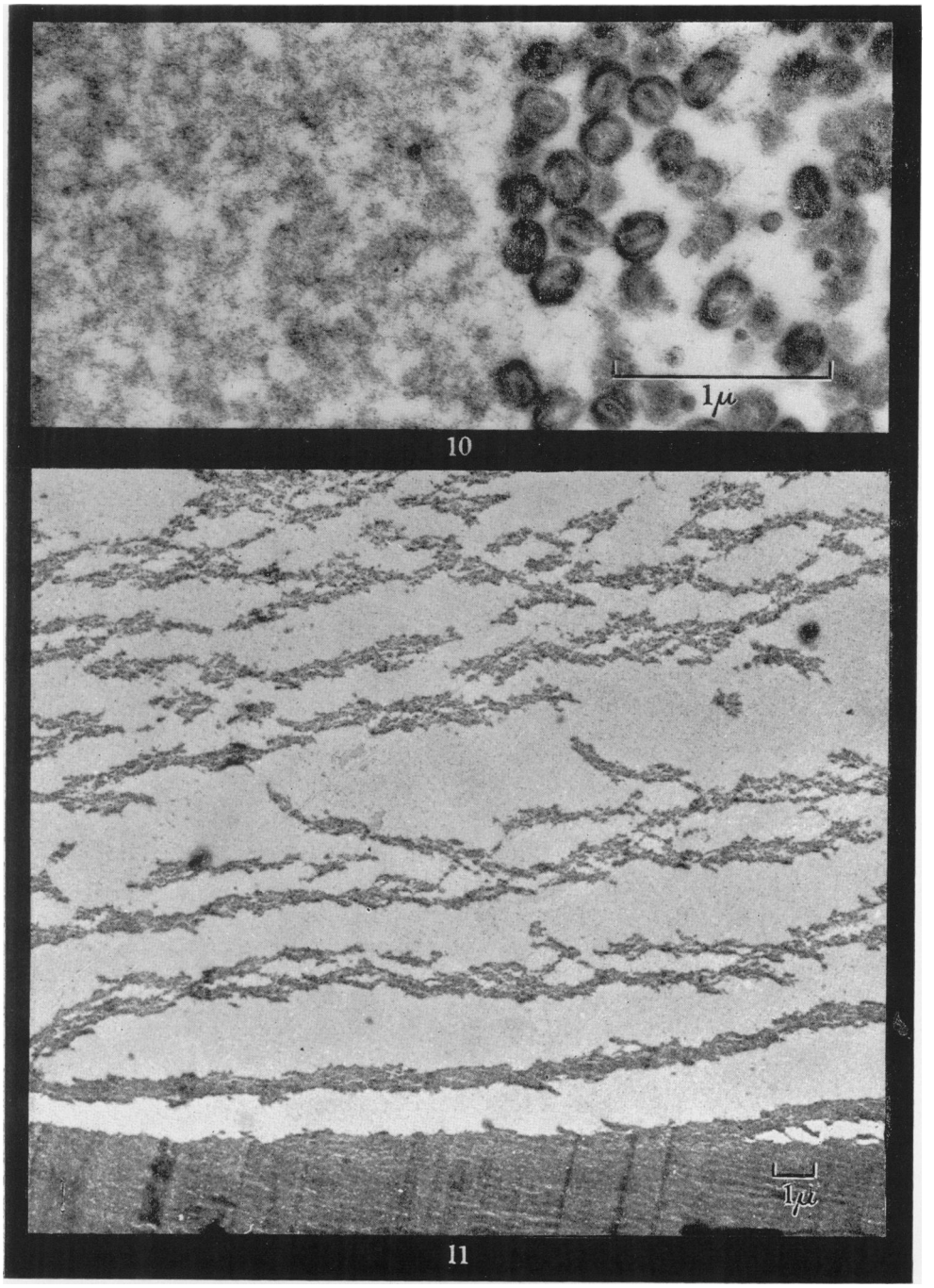

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Epstein.

observed. The particles were orientated at random and varying amounts of individual particles were present in a given section ; those particles cut tangentially had only a thin surface sliver included, in contrast to the more substantial segment taken from those cut nearer their centres (Fig. 3).

In shape, the particles resembled short, slightly flattened bricks with markedly rounded edges and ends (Fig. 2 to 7). Their fine structure consisted of an outer double limiting membrane whose two electron-dense layers lay about 5 mμ apart with a less dense layer in between (Fig. 4 to 7). This limiting membrane covered a narrow zone of slight electron density (Fig. 4 to 7) except at the centre of each of the two widest surfaces of the particles where an electron-dense lateral body was interposed (Fig. 4, 6 and 7). The narrow zone of slight electron-density was limited centrally by a further double membrane enclosing an inner area or nucleoid (Fig. 4 to 7) ; the double membrane of the nucleoid also had two electron-dense layers about 5 mμ apart separated by a less dense layer (Fig. 7). The nucleoid itself was differentiated into a central disc-shaped electron-dense region surrounded by a region whose slight electron-density resembled that of the zone lying immediately below the outer limiting membrane (Fig. 4 to 7).

Jelly, ring and tongue.—The jelly which formed the upper strata of the pellet consisted of uniform, fuzzy material (Fig. 8, 10 and 11) which had a granular appearance at higher magnification owing to the presence of fine beaded threads (Fig. 9). In areas of the pellet immediately surrounding the dense white zone, particles identical with those composing this zone were found scattered in the jelly ; the jelly lying above the dense white zone was, however, sharply demarcated from the underlying particles (Fig. 10). Above the ring and central tongue the jelly was arranged in strands forming a coarse mesh-work ; this was packed down into dense laminae in the lower layers of these regions (Fig. 11), the dense packing coinciding with the greyish opacities visible macroscopically as the ring and tongue (Fig. 1).

Combined electron microscopy and titrations

The results shown in the Table are of the 3 experiments in which the biological activity of the virus was followed during the preparation of pellets and was related to the morphological observations made with the electron microscope on samples from different regions of each pellet.

It can be seen that the infectivity of each virus suspension was very considerably greater than that of the supernatant fluid (Spinco Supernatant) above the pellet formed by high speed centrifugation of the suspension (table). After this centrifugation something of the order of 99·9 per cent of the biological activity was eliminated from the virus suspension on 2 occasions (Expt. 4 and 6) and much more than this on another (Expt. 5). That this biological activity had been deposited in the white zone of the pellets is shown by the fact that in each case a very small sample from this zone was highly active (Expt. 4, 5 and 6) even when re-suspended in as great a volume of fluid (10 ml.) as the original virus suspension from which the whole pellet came. In contrast, similarly re-suspended small samples of jelly from the centre of the pellets (Expt. 4 and 5), or from the edge (Expt. 6), possessed relatively little activity.

Samples taken from the white zones of the pellets prepared in Expt. 4, 5 and 6 were seen, when examined in the electron microscope, to be composed entirely of uniform particles having the characteristic shape and fine structure described

TABLE.—*The Biological Activity of Samples Taken at Different Stages During the Preparation of Pellets from Fluorocarbon Purified Virus Suspensions (Titration on Eggs C.A.M.)*

Expt. No.	Sample	Dilutions of sample and result: Denominator—number of live eggs harvested; Numerator—number of live eggs with lesions									LD_{50} per ml. of sample
		10^{-0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	
4	Virus suspension	—	—	—	—	4/4	4/4	3/4	0/4	0/4	$10^{-7.3}$
	Spinco supernatant	—	—	4/4	2/4	0/4	0/4	0/4	0/4	0/4	10^{-4}
	White pellet	—	—	—	3/3	4/4	2/4	0/4	0/4	0/4	10^{-6}
	Central pellet jelly	—	3/3	4/4	0/4	0/4	—	—	—	—	$10^{-3.5}$
5	Virus suspension	—	—	—	—	—	5/5	5/5	1/5	0/5	$10^{-7.6}$
	Spinco supernatant	—	—	5/5	1/5	0/4	0/4	—	—	—	$10^{-3.6}$
	White pellet	—	—	—	—	5/5	4/4	0/4	0/5	—	$10^{-6.5}$
	Central pellet jelly	—	—	5/5	3/5	0/5	0/4	—	—	—	$10^{-4.1}$
6	Virus suspension	—	—	—	—	—	4/4	4/4	1/5	0/5	$10^{-7.6}$
	Spinco supernatant	—	5/5	5/5	5/5	2/4	0/5	—	—	—	10^{-5}
	White pellet	—	—	—	—	5/5	3/4	0/5	0/3	—	$10^{-6.3}$
	Peripheral pellet jelly	5/5	4/5	0/5	0/4	0/5	—	—	—	—	$10^{-2.4}$

above (Fig. 2 to 7). Samples of the gelatinous areas of the pellets were found to be free of particles (Fig. 8, 9 and 11) when examined in this way.

DISCUSSION

The technique of fluorocarbon purification used in the present investigation is based on that described by Gessler *et al.* (1956) in their original work with compounds of this type. These workers used a mixture of fluorocarbon and *n*-heptane but noted that if Genetron 226 were the fluorocarbon chosen, *n*-heptane was not required. Genetron 226 is the trade name used in the United States for the fluorocarbon $CF_2Cl\text{-}CCl_2F$ which is sold as Arcton 63 in Great Britain. It was because of its suitability for use without the possible complicating factor of *n*-heptane that Arcton 63 was selected for the present experiments. Although 3 applications of fluorocarbon were suggested by Gessler and his colleagues (Gessler *et al.*, 1956), trials carried out in preparation for the work reported here showed that it was only the Arcton of the fourth or fifth application that became white in colour and free of material; 5 treatments with Arcton were therefore adopted as a standard procedure.

The fixative used here in preparing samples of pellets for electron microscopy was likewise chosen as a result of preliminary tests. Although buffered osmium (Palade, 1952) has been widely proved to be an outstandingly successful fixative for electron microscopy, particularly if sucrose is added to it (Caulfield, 1957), when applied to samples from the white zones of pellets prepared from purified vaccinia virus suspensions the results were disappointing. Only a narrow outer layer of the sample contained recognizable particles and not all of these were well preserved; permanganate fixation (Luft, 1956) on the other hand, gave excellent preservation of the particles right through the samples.

In the titration experiments, care was taken to ensure that the samples of material whose biological activity was to be compared were kept at a standard temperature and in the same suspending fluid until all were ready for serial dilution

and inoculation. The results obtained were further validated by inoculating the biologically feebler materials before the more active.

From the results which have been obtained it is evident that if vaccinia virus grown on the chick chorio-allantois is treated with fluorocarbon 5 times in a way similar to that suggested by Gessler *et al.* (1956), a suspension can be obtained which is entirely free of formed host cell constituents. This is shown by the fact that the pellets made by high speed centrifugation of such suspensions consisted only of regular, formed particles (Fig. 2 and 3) together with fuzzy gelatinous material (Fig. 8, 9, 10 and 11). The particles were of a size appropriate for vaccinia virus and their structure was uniform (Fig. 2 to 7). In general internal arrangement they closely resembled osmium-fixed mature extra-cellular vaccinia virus (Morgan, Rose, Ellison and Moore, 1954 ; Peters, 1956) and the results of the titration experiments (table) clearly show that the biological activity of the pellets lay in the region composed of the particles. It must be borne in mind, however, in considering the activity shown by samples from different areas of the pellets that the results obtained are not strictly quantitatively comparable, since it was not possible to cut out fragments of an exact standard weight or volume. Nevertheless, the samples were all of the same order of size whilst the differences of biological activity ranged from a factor of about 500 (Expt. 4 and 5) to a factor of 10,000 (Expt. 6). Furthermore, these results probably give an underestimate of the biological activity residing in the particles forming the dense white zones of the pellets, since in re-suspending the samples from these zones it is more than likely that the packed particles were dispersed in clumps or aggregates rather than singly.

Thus, the fluorocarbon treatment yielded suspensions in which the only formed structures were virus particles, but which contained at the same time the fuzzy material deposited by high speed centrifugation as the gelatinous portion of the pellets. That the morphological criterion of purity applied to the virus preparations was able to detect the gelatinous material says much for the method of Palade and Siekevitz (1956*a* and 1956*b*) which was used.

The presence of the gelatinous material makes it impossible to use pellets from fluorocarbon-treated vaccinia virus suspensions for chemical analysis of the virus, since the jelly, whatever its relevance, represents a contaminant. In fact, the jelly is of very great importance in this context because cytochemical studies (Holt and Epstein, 1958) have shown that it contained a substantial amount of free deoxyribonucleic acid. It is of interest to note in this connection that egg-grown Rous virus treated with fluorocarbon has been isolated with an apparently similar jelly (Epstein, 1958*c*) which likewise contained much free deoxyribonucleic acid (Epstein and Holt, 1958)

The jelly may also be of importance from a serological point of view, for in the experiments of Hamparian *et al.* (1958) viral antigens prepared from chick chorio-allantoic membranes were found to contain 2 types of reactive host components. One of these could be removed by a single treatment with fluorocarbon, whereas the other was still present in quantity even after 5 such treatments. It might be that this latter type of host component corresponds to some constituent of the jelly found in the present investigation.

The morphological observations on the mature vaccinia virus particles reported here include certain findings of interest. Thus, although the permanganate fixed particles showed a general internal arrangement similar to that described

in osmium-fixed mature extra-cellular vaccinia virus (Morgan *et al.*, 1954 ; Peters, 1956), they differed in their fine structure in some important details. Firstly, the particles always appeared plumper in the sections than those fixed with osmium (Peters, 1956) ; this was particularly evident in particles sectioned longitudinally across their shortest dimension (Fig. 4). The plumpness was probably emphasized by the use of Sotelo's (1957) technique for ensuring complete flattening and expansion of the sections, but even in preliminary work where this was not done (Epstein, 1958*a*) the particles observed were plumper and less flattened than those fixed by osmium. Secondly, the outer zone of the virus between the outer limiting membrane and the membrane surrounding the nucleoid, was narrower and less electron-dense than in osmium-fixed material. The last and most important difference was the striking differentiation of the nucleoid into an inner region of great electron-density and an outer region of slight electron-density (Fig. 4 to 7). The presence of these two hitherto unsuspected structurally distinct parts in the nucleoid is of special significance in the light of earlier enzymatic digestion studies on viruses of the pox group. For, the fact that in pepsin-treated particles the nucleoid cannot be hydrolized by desoxyribonuclease alone, but only by desoxyribonuclease followed by a further pepsin digestion, has been taken as an indication that the nucleoid contains protein as well as nucleic acid (Peters and Stoekenius, 1954). It might be that the two distinct regions found in the nucleoid of the permanganate-fixed particles are linked in some way to the presence of these two nucleoidal substances.

Although the presence of the jelly in the pellets shows that fluorocarbon treatment does not yield a vaccinia preparation containing only virus particles as had been hoped, the findings of the present work are nevertheless encouraging for a number of reasons.

Firstly, the virus has in fact been obtained so free of contaminants in the firm white zones of the pellets that separating it from the substances composing the jelly which lay around these zones is considered a feasible undertaking.

Secondly, the particles in the white zones form an extremely valuable source of easily and rapidly prepared virus material for morphological study, as the present findings on the structure of the virus indicate. Furthermore, just as permanganate was applied with ease to the fragments cut from the pellets, so other fixatives and reagents can be similarly applied. This point is illustrated by work on the composition of the nucleoid of the Rous virus (Epstein and Holt, 1958), in which samples of fluorocarbon purified pellets of this virus were treated with various fixatives and specific enzymes.

Lastly, the fact that infectivity can be shown to depend entirely on the presence of particles free of formed host cells constituents, as has been done here in the combined biological and morphological experiments, offers a most useful method for identifying unknown particles with specific viruses. This procedure has been used to confirm the nature of the particles found in association with Rous tumours (Epstein, 1958*c*) and could equally well be applied in the case of other similar virus induced conditions both neoplastic and infectious.

SUMMARY

Experiments are described which were performed to investigate the purifying effect of a fluorocarbon (CF_2Cl-CCl_2F) on vaccinia virus. The virus was grown on the chorio-allantoic membranes of chick embryos and was separated from

host cell constituents at a water-fluorocarbon interface ; the virus suspensions were subjected to a force of 105,000 *g* for 1 hr. and the pellets obtained were fixed with permanganate, embedded and sectioned for electron microscopy. Direct observation of the sectioned pellets with the electron microscope has been used as a check on their purity. In combination with the morphological work, biological tests of virus activity have been made on the material at each stage in the experiments.

The techniques which have been used are described in detail.

The results show that fluorocarbon treatment enabled virus suspensions to be made which were entirely free from formed host cell constituents but which nevertheless contained other important host substances. This was demonstrated by the fact that the pellets made by high speed centrifugation of the suspensions consisted of a firm white zone composed entirely of regular brick-shaped particles measuring about 300 mμ $\times$ 200 mμ, as well as a gelatinous region having a fuzzy, granular fine structure. The biological tests have established the viral nature of the particles in the pellets. They have shown that about 99·9 per cent of the infectivity of the virus suspensions was eliminated by the gravitational force applied and that this activity came to reside, after the centrifugation, in that part of each pellet which was composed of the particles.

A description is given of the fine structure of permanganate-fixed mature vaccinia virus particles including an account of the structural differentiation found, for the time, in the nucleoid.

Both the limitations and the possible usefulness of fluorocarbon purification of an animal virus such as vaccinia, are discussed in the light of results obtained.

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