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Strand-Length Measurements of Normal and 5-Iodo-2'-Deoxyuridine-treated Vaccinia Virus Deoxyribonucleic Acid Released by the Kleinschmidt Method

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Purified vaccinia virus, which had been grown on chick-embryo chorioallantoic membranes in the presence or in the absence of 5-iodo-2'-deoxyuridine (IUdR), was suspended in 5 M ammonium acetate and subjected to the one-step Kleinschmidt procedure on surfaces of distilled water or salt solutions. Deoxyribonucleic acid (DNA) molecules were clearly revealed, and in many instances accurate length measurements could be made. The longest continuous molecules from normal virus measured 78, 77, and 65 μ. The most frequent length was approximately 30 μ, which corresponds to only one-third to one-half of the total DNA per virus particle predicted from various chemical analyses. These data provide direct evidence that normal vaccinia DNA may occur as a linear molecule of approximately 150 $\times$ 10^6 molecular weight units, but, for reasons still unknown, the majority of these molecules appears to break into segments of equal length during release from the virion. There is no evidence for the presence of cyclic DNA. The DNA molecules are typically double-stranded. DNA from IUdR-treated vaccinia presents a markedly different picture: the molecules are mostly fragmented into small pieces, and rosettes or tangled masses equivalent to even one-quarter the length of normal molecules occur very rarely. The possibility is discussed that at least part of the virus-inhibitory effect of IUdR on vaccinia is due to extensive fragmentation of the DNA molecules into which IUdR has been incorporated in place of thymidine.

Electron-microscopic observations of deoxyribonucleic acid (DNA) strands released from several bacteriophage by the Kleinschmidt procedure (19) have provided vivid evidence not only on the lengths of the nucleic acid molecules obtained from each virus, but also on the formation of rings in normal phage DNA (16, 20, 29) or of unusual branched structures occurring in DNA replicated in vitro (11). The sensitivity of the method is such that significant differences have been demonstrated between the lengths of DNA strands obtained from wild and mutant strains of λ bacteriophage (2, 20), and heat-denatured DNA can be observed to unwind into individual strands in certain areas of a normally double-stranded molecule (10).

Recently, the Kleinschmidt procedure has been successfully applied to several small animal viruses. Cyclic DNA was clearly demonstrated from viruses of the papova group, including polyoma (2, 31), Shope papilloma (18), and human papilloma (L. Crawford, *personal communication*). The DNA of type 12 adenovirus, on the other hand, appears to be linear (6). This paper describes the application of the Kleinschmidt technique to one of the largest animal viruses, namely vaccinia. DNA from this virus has previously been seen in the electron microscope (24), but there are no published data on strand lengths. The objectives of the investigation were (i) to attempt to resolve the uncertainties about the total DNA content of vaccinia, and (ii) to investigate possible changes in DNA length or structure resulting from incorporation of the thymidine analogue, 5-iodo-2'-deoxyuridine (IUdR), into the molecule (27). While this manuscript was in preparation, an electron micrograph of fowlpox DNA was published (8), and recently another electron micrograph of fowlpox DNA, together with strand-length measurements, has appeared (9).

[1]John Falding McCrea died on 4 September 1967. He would have wished that his last paper be dedicated to the late Professore Francisco Duran-Reynals from Yale University and to Sir Macfarlane Burnet from the University of Melbourne, Australia.

MATERIALS AND METHODS

Virus. The egg-adapted Levaditi strain of vaccinia virus was used throughout. Chorioallantoic membranes of 12-day chick embryos were inoculated with approximately 1,000 pock-forming units of stock virus, incubated at 37.5 C, and harvested 40 hr later. The virus was purified by four cycles of fluorocarbon homogenization. These methods have been described in detail previously (26). Pock counts were made in the standard way (23). Pock-forming efficiency of normal purified virus was 5:1.

IUdR. The crystalline reagent (Schwartz Bio Research Inc., Orangeburg, N.Y.) was completely dissolved with stirring at a concentration of 4 mg/ml in hot (90 C) distilled water. The solution was cooled to 25 to 30 C, and then 1 ml was placed on the previously dropped chorioallantoic membranes immediately before inoculation with 1,000 pock-forming units of virus. Virus from these membranes was harvested and purified with fluorocarbon as described above. Pock-forming efficiency of IUdR-treated purified virus varied from 50:1 to 80:1.

Release of DNA. All experiments described here were carried out by a modification of the "one-step" osmotic shock method (19). After preliminary centrifugation at 1,200 × *g*, fluorocarbon-purified virus was deposited by centrifugation at 27,000 × *g* for 15 min, and the pellet was carefully resuspended in 5 or 10 M ammonium acetate to give a virus particle concentration of 10^8 to 10^9/ml. Cytochrome *c* and ethylenediaminetetraacetate (both from Nutritional Biochemicals Corp., Cleveland, Ohio) were added to the virus suspension at final concentrations of 0.5 mg/ml and 5 mg/ml, respectively. All reagent solutions were filtered through 0.45-μ membrane filters (Millipore Corp., Bedford, Mass.) before use. A sample (0.05 ml) of the virus suspension was then run down a metal ramp onto the surface of double-distilled water contained in a 9-cm diameter glass petri dish coated with paraffin wax. The surface of the distilled water had been previously "scraped" with a metal bar to remove any floating dirt particles.

After 5 to 10 min, samples were removed by touching representative areas of the surface film with electron microscope grids (stainless steel, 60-mesh) coated with

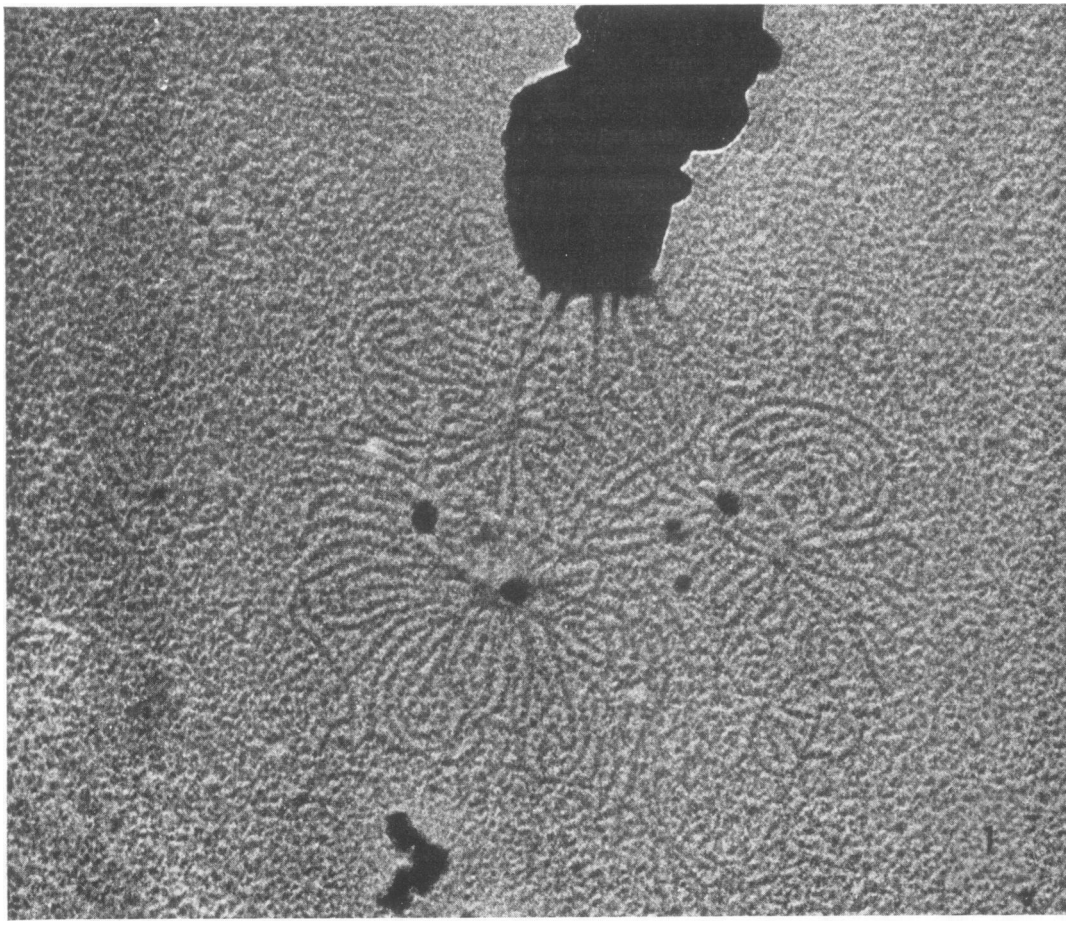

FIG. 1. *Normal vaccinia particle with DNA strands released by osmotic shock on distilled water surface. DNA appears to be escaping from several orifices.* × *110,000.*

Formvar and lightly carbon-shadowed. Between 10 and 20 grids were used in each experiment. Each grid was then dipped in absolute alcohol for 10 sec and blotted dry. Grids were placed in a Mikros VE-10 vacuum evaporator and shadowed with uranium (shadowing angle 5 to 10°) while rotating at approximately 60 rev/min on an electrically driven turntable.

Electron microscopy. Specimens were photographed in an RCA-3G electron microscope at instrumental magnifications of 7,800 or 11,800. The magnifications were repeatedly calibrated with a diffraction grating replica (E. F. Fullam, Inc., Schenectady, N.Y.) and occasionally by photographing 0.264-μ diameter polystyrene latex balls (Dow Chemical Co., Midland, Mich.). Particle counts were made by mixing equal volumes of virus and 0.264-μ latex balls (suspended in 0.1 M ammonium acetate containing 0.02% crystalline bovine serum albumin), sonic treating for 3 min to disrupt virus clumps (MSE Ultrasonic Disintegrator, 60 w), spraying microdroplets from a modified

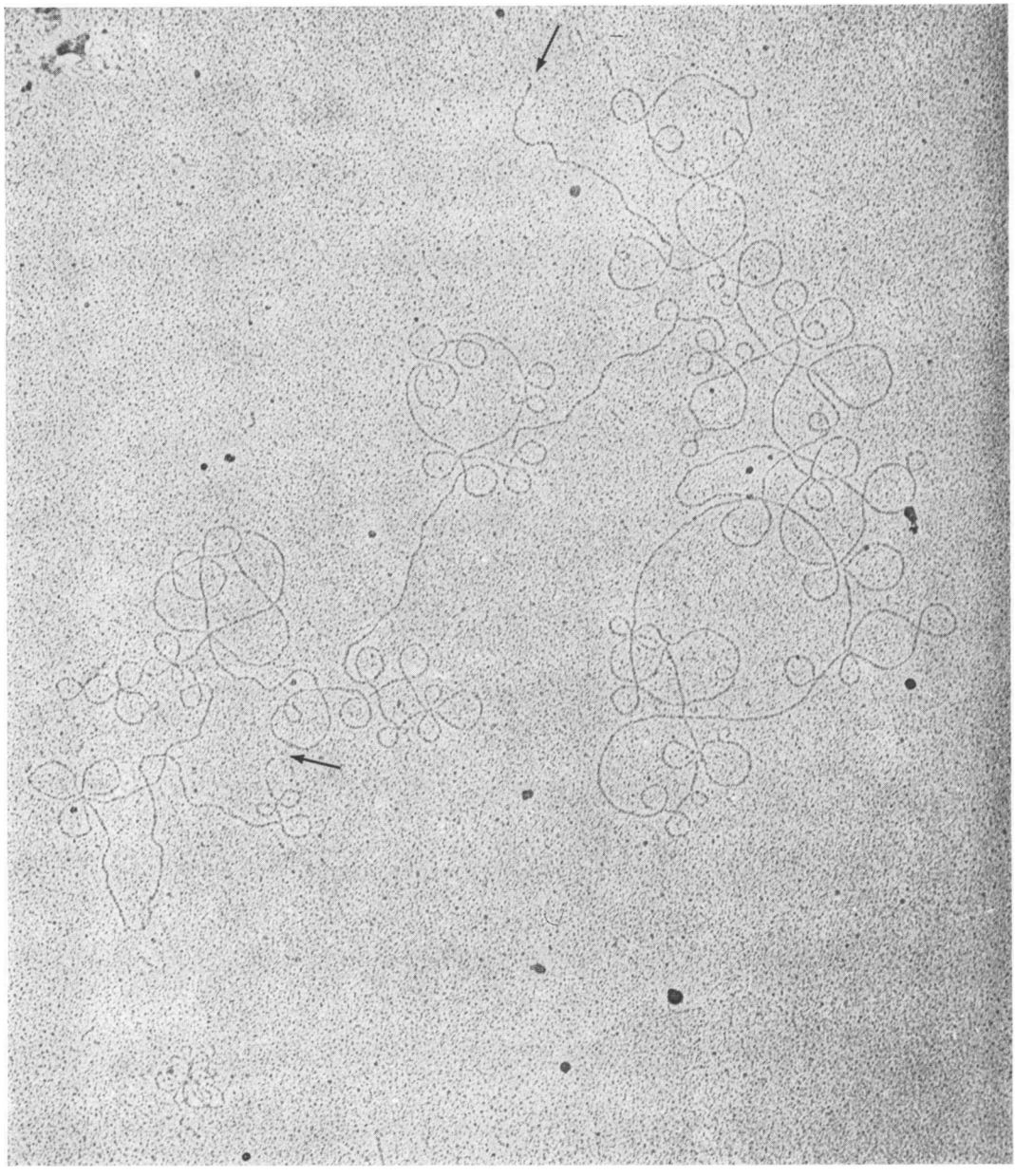

FIG. 2. *Continuous DNA molecule from normal vaccinia with two free ends (indicated by arrows): total length 46 μ. × 30,400.*

De Vilbis sprayer onto carbon-coated grids, shadowing with platinum-carbon, and photographing the droplets in the electron microscope.

Measurement of DNA strands. Suitable electron microscope plates were projected onto paper at a magnification of 10 and traced with pencil, and the lengths of the strands were measured with a calibrated map measurer (Dietzgen, Switzerland). Tracings were made and measured independently by two observers. Conversion of DNA strand lengths to molecular weight units was made by multiplying the strand length in microns by the factor 1.92×10^6 (20).

RESULTS

DNA strands from normal vaccinia. Preliminary experiments with relatively concentrated virus preparations (about 10^9 particles/ml) showed clearly that vaccinia consistently released a large portion of its DNA when treated by the one-step process from 5 M ammonium acetate onto a distilled-water surface. In many instances, the DNA strands seemed associated with the remains of virus particles (Fig. 1). Subsequent experiments were carried out at lower initial virus concentrations (10^8 particles/ml), and the virus was subjected to the Kleinschmidt procedure within at most a few hours after harvest and purification. The majority of particles spread on distilled water now revealed somewhat more open arrays of DNA (Fig. 2). Of several hundred virus particles photographed and examined, only three showed apparently continuous strands of DNA that approached the length expected for the mass of DNA per particle calculated from chemical analysis. Figure 3 illustrates one of these. Two ends

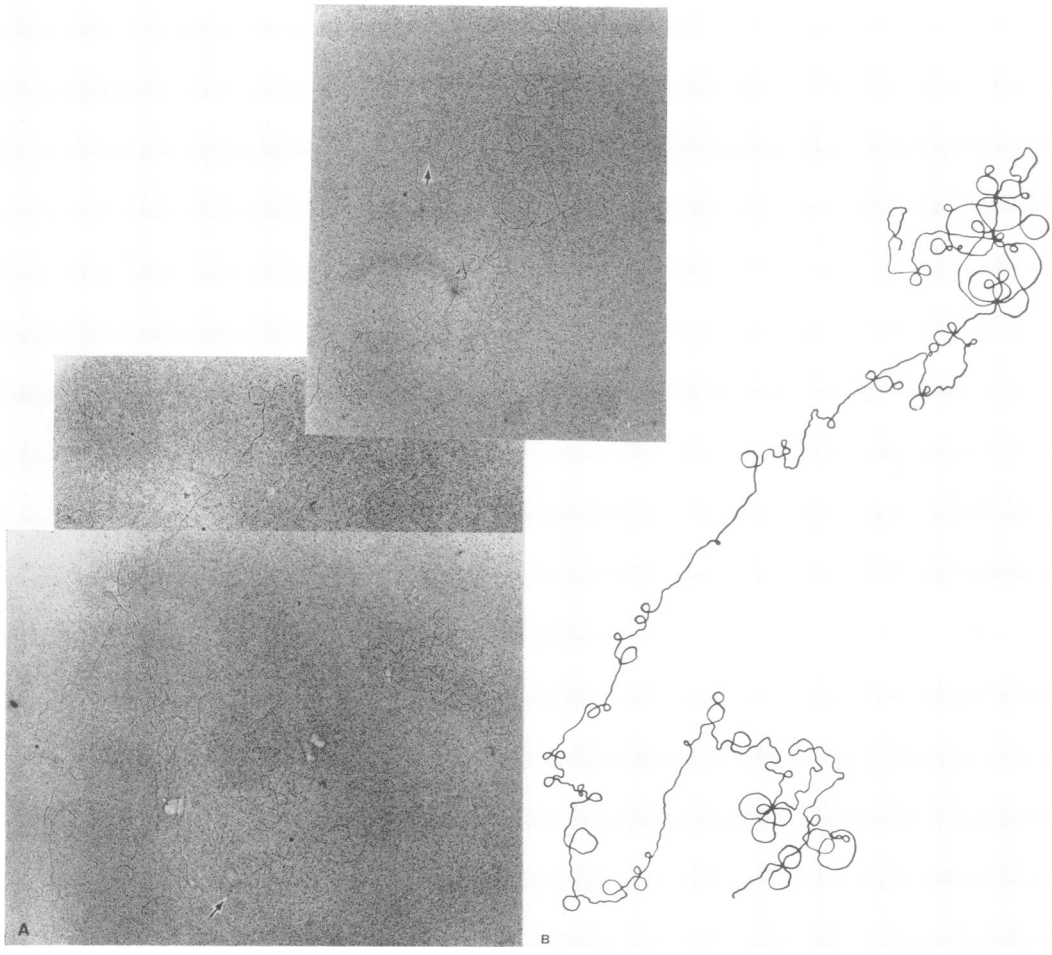

FIG. 3. *Three electron microscope plates and tracing of a continuous DNA molecule, 78 μ long, from normal vaccinia.* × *21,000.*

are clearly visible on a total strand length of 78 μ.

A histogram of strand-length measurements is presented in Fig. 4. The three longest strands were closely similar in length (78, 77, and 65 μ). The most frequently occurring strand lengths above 10 μ are between 20 and 40 μ (mean 30 μ). No attempts were made to measure the numerous fragments less than 10 μ long.

DNA strands from IUdR-treated vaccinia. Purified vaccinia grown in the presence of 4 mg of IUdR per egg was suspended in 5 M ammonium acetate (10^8 particles/ml) and spread on distilled water in exactly the same way as described for normal virus. Each experiment with IUdR-treated virus was run in parallel with a preparation of control virus from membranes inoculated at the same time with the same virus dilution, but previously inoculated with 1 ml of distilled water instead of 1 ml of IUdR solution. The electron microscope grids from normal and IUdR-treated virus samples were taken from similar representative areas of the water surfaces, placed on the same glass slide, and then shadowed simultaneously.

Figure 5 illustrates a typical example of the appearance of an IUdR-substituted vaccinia particle and associated DNA strands. Several hundred of these particles were observed in different experiments. In the great majority of microscope fields, the DNA appeared as small random fragments, distributed widely over the field, difficult to visualize, and often in small, tangled masses or in rosettes (Fig. 5). Many of the shorter fragments were characteristically zig-zag, showing sharper bends than DNA from control virus. Meaningful measurements of IUdR-treated virus DNA were difficult to obtain because of the extreme and irregular fragmentation. Long continuous strands like those obtained from control virus (Fig. 3) were, however, never seen. The IUdR-substituted DNA appeared typically double-stranded.

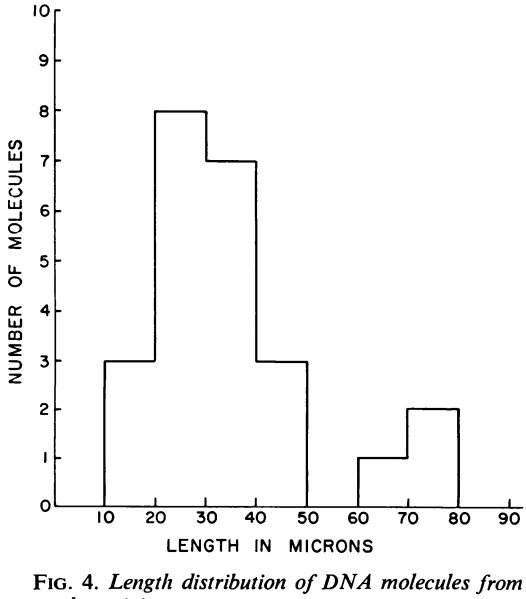

FIG. 4. *Length distribution of DNA molecules from normal vaccinia.*

DISCUSSION

The size of the DNA molecule in poxviruses has not been unequivocally established, despite the fact that these viruses have been subjected to repeated chemical analyses for almost 30 years (7, 25). The DNA content reported by several workers is 5.6 to 7.8% (1, 7, 12, 15, 25), varying only slightly between different poxvirus strains (12). The dry weight of the vaccinia particle is 5.3×10^{-9} to 5.5×10^{-9} μg (1, 12, 30), and the mean weight of DNA per particle is reported to be approximately 2.6×10^{-10} μg (1, 30). Consequently, one viral particle would be expected to contain about 160×10^6 molecular weight units of DNA (1, 12, 13), which is equivalent to a strand length of approximately 84 μ for double-stranded DNA in the B configuration (20). The most recently reported DNA content of vaccinia is, however, significantly lower, being 3.2% for virus grown on rabbit skin and purified on sucrose density gradients (33); this is equivalent to 1.8×10^{-10} μg of DNA per particle, a molecular weight of 110×10^6, and strand length of about 55 μ. There is thus a more than twofold difference between the highest (1) and lowest (33) published figures for the DNA content of vaccinia.

Sedimentation data for cowpox DNA indicate a homogeneous population of molecules which are, however, at most only one-half of the expected molecular weight (13). The molecular weight of cowpox DNA calculated from the sedimentation coefficient of 47 was reported to be 80×10^6, but this is a maximal estimate because of the type of sedimentation equation used (13); a more accurate value, by use of the recent data of Eigner and Doty (4), would be 60×10^6. The "molecular weight" of the infectious unit of vaccinia estimated from rates of inactivation of infectivity by γ rays or high-energy electrons is about 40×10^6 (21, 32).

The experiments with vaccinia reported in this paper provide evidence for the existence of linear DNA molecules up to 78 μ long (Fig. 3). The DNA appears to be double-stranded. Strand length of 78 μ is equivalent to a molecular weight of about 153×10^6, or 95% of the maximal value predicted from chemical analysis. Molecules this long have been observed very rarely, but it seems unlikely that they are artifacts produced by end-

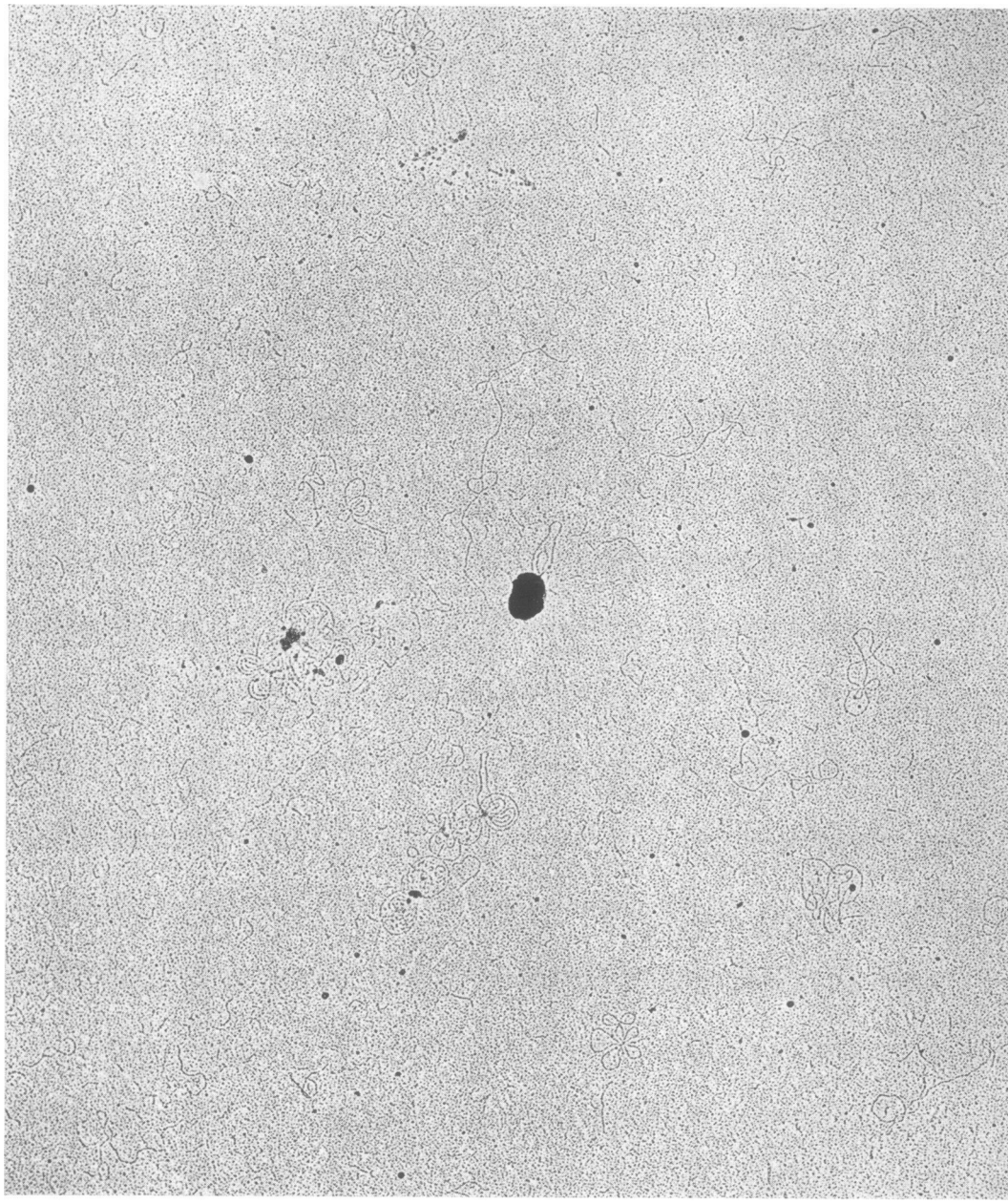

FIG. 5. *IUdR-treated vaccinia. DNA molecules characteristically fragmented into short pieces and small rosettes.* × *32,000.*

to-end arrangement of two shorter molecules. Inspection of electron micrographs alone cannot, however, entirely eliminate this possibility (20). Phage λ DNA may be seen in electron micrographs to exist in linear or cyclic form through the joining of two cohesive ends (16, 20), but there is as yet no electron microscopic or sedimentation evidence for the existence of similar "sticky ends" or of cyclic DNA in poxviruses.

The most frequently observed length of vaccinia DNA released by osmotic shock in the present experiments is about 30 μ (Fig. 4), equivalent to a molecular weight of about 60 × 10^6. This agrees closely with the corrected molec-

ular weight (5) of 60 × 10^6 calculated from the sedimentation coefficient of cowpox DNA extracted with 2-mercaptoethanol and papain (13). The relationship of these released molecules to the intact DNA within the virion could be explained in two possible ways. (i) If the most recent chemical analysis of vaccinia DNA (33) is correct, the molecular weight of the DNA is approximately 110 × 10^6 and the majority of the released molecules would represent half molecules. (ii) If earlier analyses are correct, the molecular weight of the DNA is 160 × 10^6, and the majority of the released molecules would most likely represent one-third molecules. Since we have observed DNA of molecular weight up to 153 × 10^6 in electron micrographs, this latter possibility seems the more likely. For reasons at present unknown, it is obvious that vaccinia and cowpox virus DNA is exceptionally liable to shear during release from the virion, possibly because of some unusually strong linkage at one or two sites between the DNA and structures within the viral particle. The existence of more than one DNA molecule within each poxvirus virion is unlikely on genetic grounds, since in rabbitpox at least 18 mutants can be arranged in linear order in a way analogous to the arrangement of r mutants in T-even phage (5).

Virus inhibitory activity of IUdR may occur at various stages during virus replication, as for example with enzymes concerned in early stages of DNA synthesis, in the final assembly of the DNA and protein components, or in the transmission of correct information from halogen-substituted viral DNA to progeny virus (17, 28). About 15 to 20% of vaccinia DNA-thymidine is replaced by IUdR when the virus is grown under the conditions described in this paper (27). Detailed examination of this substituted DNA reveals only minimal density changes; there is no change in the thermal transition temperature of the DNA, nor is the sensitivity of virus to either ultraviolet or γ-ray inactivation increased (Berrah and McCrea, *unpublished data*).

The electron micrographs of IUdR-substituted vaccinia DNA show a striking change when compared to normal vaccinia DNA. In every experiment, the substituted DNA was fragmented into small random pieces, and continuous strands as long as even one-quarter molecules were very rarely seen (Fig. 5). It should be stressed that each sample of IUdR-treated virus was run in parallel with control virus that was grown in the same batch of eggs, inoculated with samples from the same virus dilution, and purified and processed for electron microscopy at the same time. A control for the IUdR-treated virus in Fig. 5 is shown in Fig. 3; the only experimental difference between the preparations illustrated in the two figures is that the eggs yielding the virus in the former received 1 ml of distilled water before inoculation, whereas the latter received 1 ml of IUdR solution.

It seems probable that the decreased pock-forming efficiency of the IUdR-substituted particles is at least partly due to the increased fragility and fragmentation of the viral genome, which could occur either before assembly into mature virion, or when the DNA is released from the virion by osmotic shock or, in vivo, by the "uncoating" enzymes of the host cell (14). From sedimentation data, it has been proposed that 5-bromodeoxyuridine-substituted vaccinia DNA may be fragmented within the viral particle (3), and it is possible that IUdR substitution of vaccinia leads to similar fragmentation of the DNA before assembly. From the present data it is not possible to decide whether the extreme fragmentation of IUdR-substituted vaccinia DNA has already taken place before assembly in the virus particle or whether it occurs by shearing of the molecules as they are released. Such fragmentation should, however, be reflected in the sedimentation and viscosity of the DNA, factors which are currently being investigated.

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