PHYSICAL STUDIES ON POX VIRUSES

I. Inactivation of Vaccinia Virus Infectivity with Low-Energy Electrons

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ABSTRACT Vaccinia virus was irradiated *in vacuo* with low-voltage electrons of restricted ranges. It was found that the pock-forming ability of the virus was not decreased after bombardment with electrons penetrating 100 A beneath the virus surface. There was very slight reduction in titer with large doses of electrons penetrating 330 A, but a sudden marked drop in infectivity occurred after exposure to electrons penetrating 500 to 700 A. Electrons of higher energies, including those capable of penetrating the virus particle completely, did not produce significant further fall in infectivity titer. It is concluded that a highly radiation-sensitive unit essential for pock formation is situated 500 to 700 A beneath the surface of the virus particle, possibly in the form of a shell. The relation of this finding to the known structure of the virus and to other radiation data on the dimensions of the infectious unit is discussed.

INTRODUCTION

Vaccinia was the first animal virus inactivated with ionizing radiations. Exponential loss of infectivity following exposure to x-rays was demonstrated in 1939 by Gowan and Lucas (1), and similar inactivation curves were obtained shortly afterwards by Lea and Salaman (2) in more detailed experiments with x-rays, gamma rays, and alpha particles. The virus has been studied recently with high-energy ionizing particles from several radiation sources, and from the slope of the inactivation curves so obtained it has been concluded that the infectious unit may be represented as a sphere approximately 500 A in diameter (3).

In this paper we describe experiments in which dry vaccinia virus was exposed to low-energy electrons of restricted ranges; *i.e.*, electrons accelerated to velocities sufficient to penetrate to predetermined depths. Such particles have previously been

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used to study the intracellular distribution of enzymes in yeast (4, 5), and also to investigate structures within bacterial spores (6, 7). The T_1 bacteriophage (8) and Newcastle disease virus (9) have been shown to possess radiation-insensitive "skins" 200 to 500 A thick by means of low-energy electron bombardment.

Because of its large size and because of the complex structures apparent in thin sections of the virus particle examined with the electron microscope (16, 17), vaccinia virus seemed particularly suitable for "partial virus irradiation" with low-energy electrons, with the aim of discovering which zone, if any, was particularly associated with the property of infection and consequent pock formation. It also seemed valuable to compare such results to those obtained from inactivation of infectivity with high-energy electrons and other particles. A preliminary account of some of these experiments was included in a general review of the effects of ionizing radiations on animal viruses (3).

MATERIALS AND METHODS

Bombardment. The accelerator used in all these experiments has been described previously (4). In essence the machine consists of a vacuum chamber 28 inches in diameter, in which stainless steel discs (½ inch diameter) covered with material to be irradiated are arranged in concentric rings; electron guns rotating above the discs deliver a homogeneous electron beam which is recorded by a galvanometer. Dose is determined by the number of revolutions of the electron guns over the target. At least ten discs were used for each assay point, and groups of controls were placed in the vacuum chamber, but out of range of the electron beam, in each experiment.

Virus Preparation. The Levaditi strain of vaccinia virus, which had been through at least 15 consecutive passages on the chorioallantoic membrane of 12-day chick embryos, was used throughout. Embryos were inoculated with 0.1 ml of virus diluted 10^{-3} to 10^{-4}, and the membranes removed after incubation at 37° for 48 hours. Membranes showing semiconfluent pocks were cut finely with scissors and then homogenized thoroughly with glass TenBroeck homogenizers, allowing 1 ml of normal saline buffered with M/100 pH 7.1 phosphate buffer for each membrane. Aliquots of the homogenate were frozen at −20° in small sealed tubes, and thawed and centrifuged at slow speed immediately before use.

In preliminary experiments, infectious particles were deposited from the membrane homogenate by centrifugation for 1 hour at 30,000 *g*, washed once with distilled water, and resuspended in the original volume of distilled water. All these procedures were carried out at 4°C. The virus shows little or no significant loss in infectivity after exposure to distilled water, as observed previously by Hoagland, Smadel, and Rivers (10), and is in fact resistant to inactivation by osmotic shock from 10 per cent NaCl into distilled water (11). However, freeze-drying of the washed virus on stainless steel discs by various methods gave highly irregular and unpredictable infectivity titers, and on occasion the titer dropped 2 to 3 logs. Similar difficulty was experienced with dry Newcastle disease virus on stainless steel (9). In spite of variations in freezing and drying techniques, we were unable to obtain consistent high-titer dried virus after centrifugation and washing in distilled water. Since it seemed important to irradiate virus which was as undamaged as possible by preliminary treatment, further experiments were performed in

which virus-containing homogenate was dialyzed against distilled water at 4° overnight, diluted 10^{-2} in distilled water, and then lyophilized on steel discs. Loss of infectivity was slight after these procedures, being at most 50 per cent of the untreated virus titer, and in the majority of experiments considerably less. Although such dried virus is undoubtedly contaminated with materials from the host tissue, the inactivation curves obtained below suggest that the contamination was without significant effect.

The exact procedure finally adopted was as follows: Infected membranes were washed and homogenized in distilled water, diluted 10^{-2} or 10^{-3} in distilled water, and 0.02 ml volumes pipetted onto scrupulously clean stainless steel discs. The virus was frozen on the discs at −20°, and then transferred to a chilled desiccator which was connected to a high vacuum pump for 2 to 3 hours. When dry, the virus suspension formed a uniform, barely visible opalescent film over the entire disc surface; granular or irregular patches over the surface were usually due to improperly cleaned discs. Such discs were discarded. When drying was complete, the discs were rapidly transferred to the irradiation chamber, which was then evacuated. After irradiation the discs were removed and each group of ten immersed in 2 ml of buffered pH 7.1 saline containing 1000 units of penicillin and 0.1 mg of streptomycin/ml. The tubes containing the discs were allowed to stand for 20 minutes at 4° with occasional shaking. The resuspended virus was then diluted in 10-fold steps, and 0.1 ml volumes rapidly inoculated into groups of at least six 12-day chick embryos for each dilution. Pock counts were made after incubation at 37° for 48 hours, the end point being taken at the dilutions which showed an average of 20 to 30 pocks per membrane (12). With careful standardization of all steps described, the titer of resuspended control virus was remarkably uniform in all experiments, the range of activity being from 2×10^7 to 8×10^7 pock-forming units (PFU) per ml.

RESULTS

Dose-survival curves for vaccinia virus irradiated with electrons of six different energies are shown in Figs. 1 to 4. In each experiment virus activity is plotted as per cent of the titer of controls which were shielded from the electron beam but otherwise treated identically. Different symbols on each curve represent data from different experiments.

Fig. 1 shows that no decrease in infectivity occurs after exposure to 500 volt electrons, even at the highest doses (16×10^{13} electrons/cm^2). Further experiments are required to show whether or not the slight upward curvature is statistically significant. A similar "activating" effect has been observed when this virus was exposed to low doses of electrons in other types of accelerators (11). One thousand volt electrons (Fig. 2) cause considerable scatter in the data, with a possible loss of infectivity at the highest electron dose.

The inactivation curve obtained after exposure to 1500 volt electrons (Fig. 3) shows an immediate drop in infectivity, at first exponential with dose, but then flattening at approximately 10 per cent of the control titer. Essentially similar curves are obtained with 2000 and 2500 volt electrons (Figs. 3 and 4). Only 4×10^{13} electrons/cm^2 are required to reduce survival to approximately 2 per cent of the controls when the electrons are accelerated by a 5000 volt field (Fig. 4).

The electron ranges given in Table I are approximately the average between those obtained experimentally by Davis (13) and those calculated by Lea (14). The former author has discussed the question of electron ranges in biological material in considerable detail (7, 8, 13), as has also Preiss (4, 5). Admittedly such ranges are not known precisely, but the reader who consults the above references will see that the agreement between experiment and theory is quite good. In most of the experiments involving such range-restricting techniques it has been the *form* of the property survival-electron energy (and therefore range) function which has been of primary importance.

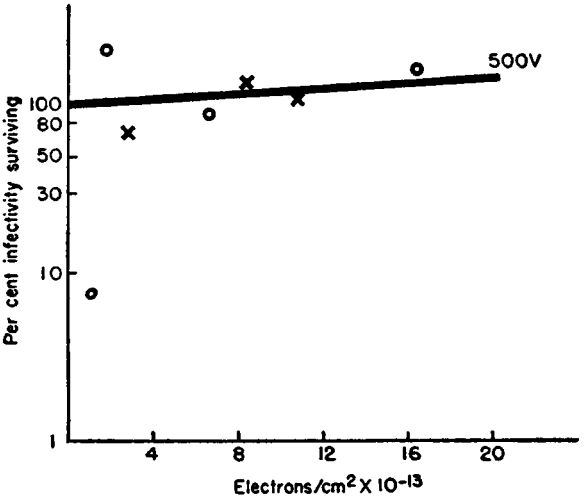

FIGURE 1 Effect of irradiating vaccinia virus with 500 volt electrons (penetration to 100 A).

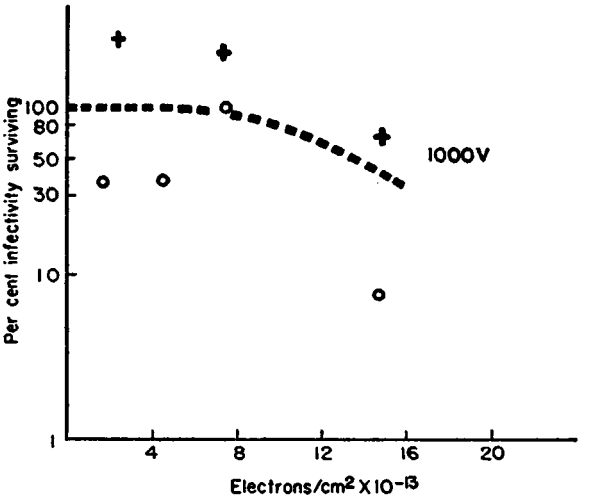

FIGURE 2 Effect of irradiating vaccinia virus with 1000 volt electrons (penetration to 330 A).

DISCUSSION

The property under study is clearly a step function of electron energy, for a sharp downward break to about 10 per cent survival occurs at 1500 volts. When a beam of mono-energetic particles is stopped in an absorbing material, there can be only an average trajectory length, for they do not all experience the exact same number of inelastic collisions while traversing a given mass of absorber. This phenomenon is called "straggling" (5), and is one of the factors which guarantees that the above mentioned step can never be a perfect discontinuity. Another more serious factor is the position of the virus particles on the bombardment discs, both relative to one another and contaminating material. In fact it could be argued that the lower-energy electrons produce no inactivation because they cannot penetrate a layer of chorioallantoic membrane and salt residues which shields the virus particles. This layer

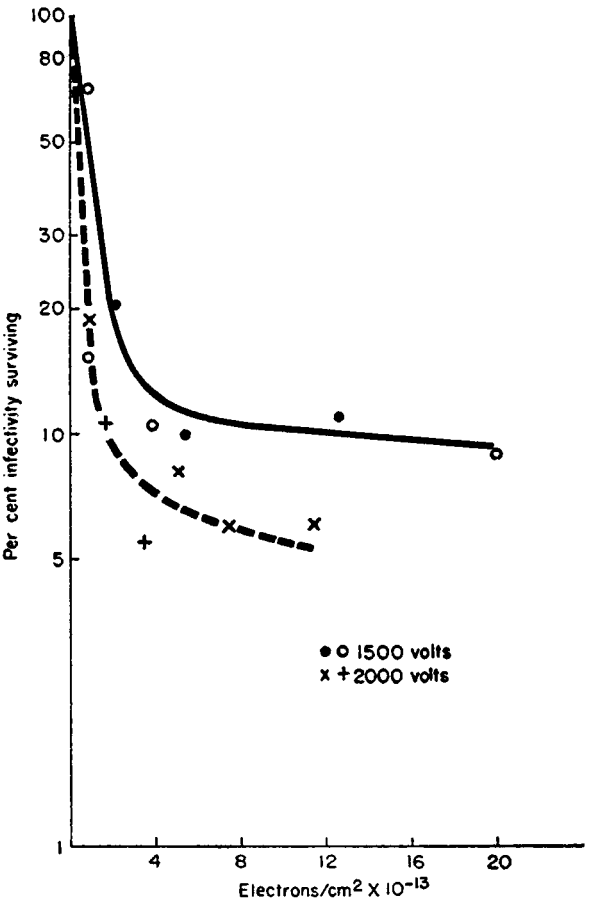

FIGURE 3 Effect of irradiating vaccinia virus with 1500 volt electrons (penetration to 700 A) and 2000 volt electrons (penetration to 1200 A).

would have to be both omnipresent and uniform, for a threshold energy exists for its penetration. It could only satisfy both of these requirements by being attached to every virus particle, for all survive 500 volt electron bombardment. On the other hand, if a conglomerate mass of virus particles and contaminating materials was on the irradiation discs, we would not expect to see the near discontinuity in the survival-electron energy function. Now the only entities on the discs which we can be sure have uniform structures are the virus particles themselves. From these considerations it is reasonable to conclude that (*a*) all virus particles have a shielding

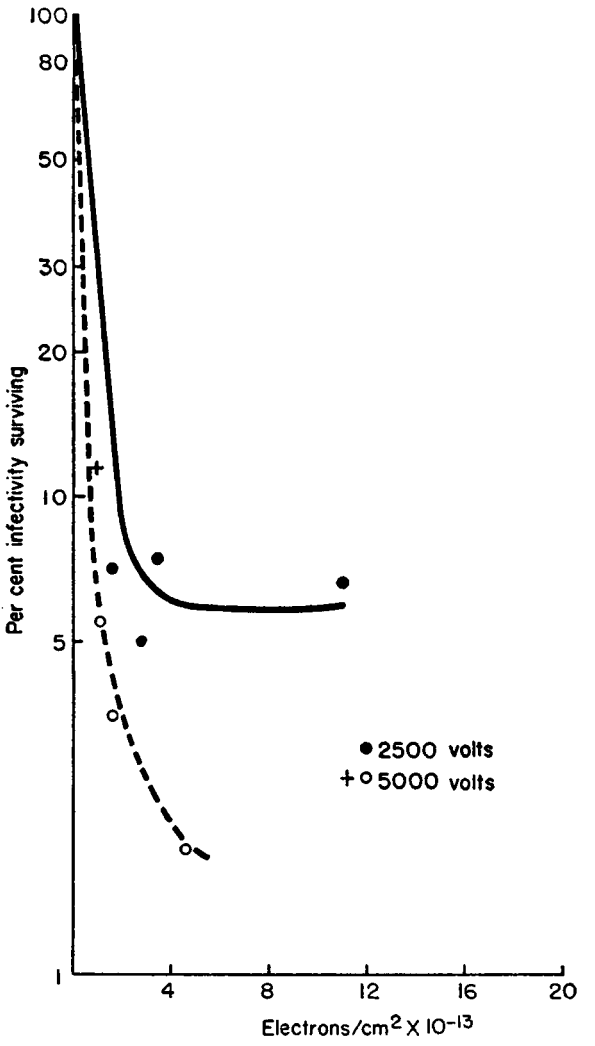

FIGURE 4 Effect of irradiating vaccinia virus with 2500 volt electrons (penetration to 1750 A) and 5000 volt electrons (penetration to 5700 A).

TABLE I

THE MEAN RANGE OF ELECTRONS IN ORGANIC MATERIAL

These are the averages of those obtained experimentally by Davis(13) and those calculated by Lea(14). Above 2000 volts they are essentially those of Lea, at density 1.3.

Energy	Range
ev	*A*
500	100
1000	330
1500	700
2000	1200
2500	1750
3000	2400
4000	3900
5000	5700

coat; (*b*) this coat is of the same thickness for all virus particles; (*c*) this coat is an integral part of the structure of a virus particle.

The 10 per cent activity remaining after bombardment with 1500 volt electrons, and approximately 6 per cent remaining after the same treatment with 2000 to 2500 volts, probably represent that portion of virus shielded by other virus particles, salt, or other contaminating substances from the membrane suspensions. The 5000 volt electrons which can easily traverse two overlapping virus particles eliminate most of this remaining activity. The faint inactivation produced by 1000 volt electrons is probably the result of straggling of electrons beyond the mean range (about 300 to 330A), since enormous doses are required to see it.

The data presented show clearly that ionizations from high doses of electrons hitting the surface of dry vaccinia particles, or penetrating as far as 330 A below the virus surface, cause little or no change in the ability of the virus to form pocks on the chorioallantoic membrane. Ionizations occurring 500 to 700 A or more beneath the virus surface, however, cause rapid and significance loss of infectivity. These results suggest that the virus possesses a relatively radiation-insensitive "skin" approximately 500 to 700 A thick, beneath which is situated a highly radiation-sensitive infectious unit.

The data from all experiments are summarized in Fig. 5, in which the inactivation curve is plotted on an arithmetic scale; beneath this curve is a schematic section of a vaccinia virus particle drawn on the same scale. The inactivation curve shows that maximum loss of infectivity occurs when electrons penetrate 500 to 700

A beneath the virus surface, and that deeper penetration (including those electrons capable of traversing the whole particle) produces no further significant fall in infectivity. That inactivation is most rapid and extensive over the electron penetration range between 330 and 750 A, and does not increase significantly with electrons ionizing deeper in the nucleoid region, suggests that an ionization on the outer edge

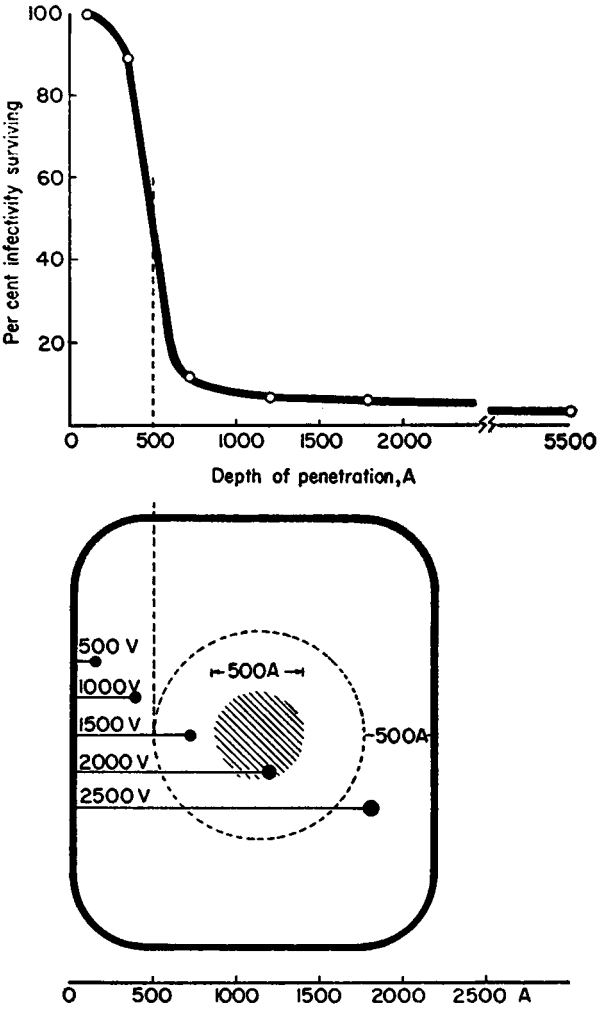

FIGURE 5 Inactivation of vaccinia virus infectivity plotted as a function of depth of electron penetration. The curve is drawn above a schematic outline of vaccinia particle on the same scale; the depth to which electrons of various voltages penetrate is indicated schematically. It should be noted that ionizations occur at random along the electron paths, and that the paths themselves are in practice not perfectly straight. The central cross-hatched circle represents the dimensions of the radiation-sensitive infectious unit calculated from bombardment with fast particles. For details see text.

of the infectious unit is capable of inactivating the whole unit. Previous inactivation experiments with high-energy ionizing particles have also indicated that a single ionization anywhere within a viral infectious unit may inactivate the whole unit (2, 14, 15). From the present experiments the outer bound of this unit is perhaps best defined from the mid-point of the steep slope of the inactivation curve, as illustrated in Fig. 5, and would thus be approximately 500 A beneath the surface, as represented by the dotted circle. Slow-electron data alone do not in this case allow one to distinguish whether the radiation-sensitive zone is in the form of a shell, or represents the outer bound of a solid core.

Irradiation of the same strain of vaccinia *in vacuo* with high-energy electrons from a Van de Graaff generator, or with gamma rays from a cobalt-60 source, gave exponential loss of infectivity down to approximately 1 per cent of the control titer. From the slope of these curves it has been calculated that the volume of the radiation-sensitive infectious units is 6.7×10^{-17} cc, which is equivalent to a sphere of approximately 500 A diameter (3). If such a sphere was located in the center of a dry vaccinia particle (2600 x 2200 A) the electrons would have to travel at least 850 A to reach the outer boundary of the sensitive region (Fig. 5). This distance, calculated from target theory (14, 15) and the geometry of the virus particle, is thus greater than that indicated by the experimental results obtained in this paper showing rapid inactivation by slow electrons penetrating 500 to 750 A beneath the virus surface. A centrally located sphere, 500 A below the virus surface, would be 1100 A in diameter, and would thus contain approximately 10 times the volume of the radiosensitive infectious unit calculated from inactivation with high-energy particles (3). This difference in the volume calculations would suggest that the radiosensitive material may be in the form of a shell, 6.7×10^{-17} cc in volume, the outer bound of which is approximately 500 A beneath the virus surface. It should be noted however, that the estimates of the electron ranges used in our experiments were originally made for proteins, and that the ranges in the outer virus coat composed of unknown proportions of proteins and lipoproteins may be slightly different.

One of the main interests in using range-restricted electrons with a virus of the complexity of vaccinia is in correlating results with the structures seen in preparations of the virus examined in the electron microscope. It has been demonstrated in electron micrographs that vaccinia contains a "nucleoid," which is susceptible to desoxyribonuclease after suitable preliminary treatments (16). Recently, Epstein (17) obtained excellent electron micrographs of ultrathin sections of vaccinia purified with a fluorocarbon, showing that the nucleoid consists of a central electron-dense discoid body and an outer region of low electron density, the whole surrounded by double membranes. Essentially similar pictures of vaccinia purified with fluorocarbon and sectioned in our laboratory show that the distance between the outer viral membrane and the double membrane surrounding the nucleoid is 350 to 400 A. At present it would be rash to draw too close comparisons between this dis-

tance and the schematic outline of the virus based on radiation experiments with slow electrons (Fig. 5), but it may be observed that the concept of virus structure obtained by two entirely different techniques is not dissimilar.

The infection of a host cell by a virus, as measured by the subsequent development of specific lesions, is a complex process dependent on several successive stages. Theoretically it should be possible to prevent the development of lesions by suitable blockade at any one of these stages. For example, the surface of the virus particle may be modified in such a way that it is unable to atach to or enter the host cell. The extremely rapid and marked loss of infectivity, concomitant with distortion of the virus surface, which follows momentary contact of vaccinia virus with concentrated urea or guanidine hydrochloride probably represents this type of inactivation (18, 19). It is extremely unlikely, however, that changes in the virus surface or outer "skin" are implicated in the present experiments, because ionizations from heavy doses of electrons penetrating up to 330 A did not significantly reduce pock-forming ability. It might be argued that since one side of all virus particles was fixed to the steel discs, and hence could be reached only by electrons penerating through at least 2200 A of virus body, all particles (except those bombarded with electrons in the 3000 to 5000 volt range) would have approximately 20 per cent of their surface untouched by electrons, and that this intact portion of the surface could be sufficient to initiate infection. Inspection of the inactivation curve in Fig. 5, however, makes this explanation extremely unlikely because there is clearly no further significant reduction in infectivity with the higher voltage electrons which are capable of penetrating through the under surface of the virus particles.

It would seem that low-voltage electrons are a useful tool in the study of large viruses such as vaccinia. Further experiments are planned to investigate surface structures such as antigens and hemagglutinins of vaccinia and other pox viruses.

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