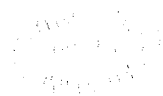
THE EFFECTS OF ULTRAVIOLET RADIATION ON VACCINIA VIRUS

P. M. Abel

A thesis submitted for the degree of
Doctor of Philosophy in the
Australian National University.

1961



G 35200

Statement

The experimental data presented in the three papers prepared for publication and in the Appendix are my own original work.

The infective centre experiment with Vaccinia-Mill Hill (VMH) virus in KB cells, described in the Appendix, was done in co-operation with Mr. K.B. Easterbrook, who infected the KB cells and made the fluorescent antibody counts.

My role in the paper by Joklik, W.K., Abel, P., and Holmes, I.H. (1960). Nature 186, 992-993, was conceptive, Dr. Joklik and Mr. Holmes carrying out the experimental proof of the idea.

P. M. Abel.

CONTENTS

Statement.

INTRODUCTION

ORIGINAL INVESTIGATIONS PRESENTED AS PAPERS
PREPARED FOR PUBLICATION

Paper I.

The Effects of Ultraviolet Radiation on Vaccinia Virus. 1. Inactivation, Mutation, and Marker Rescue.

Paper II.

Topography in Vaccinia Virus Genetics.

Paper III.

The Effects of Ultraviolet Radiation on Vaccinia Virus. 2. Multiplicity Reactivation.

GENERAL DISCUSSION.

APPENDIX.

I. Virus and Infective Centre Assays.

II. Inactivation Experiments.

III. Joklik, W.K., Abel, P., and Holmes, I.H. (1960). Reactivation of Poxviruses by a Non-Genetic Mechanism. Nature 186, 992-993.

Acknowledgements.

BIBLIOGRAPHY

INTRODUCTION

Recent technical advances in animal virology and the development of the field of vaccinia virus genetics (Fenner and Comben, 1958; Gemmell and Cairns, 1959; Gemmell and Fenner, 1960) have made possible a systematic investigation of the effects of ultraviolet radiation on an animal virus. The results of such a study are reported here.

The first section of the thesis is an Introduction on the properties of ultraviolet radiation and their relationship to the study of viruses. Three papers are then presented which describe the essential findings on the effects of ultraviolet radiation on vaccinia virus. These are followed by a General Discussion in which a comparison is made of the radiobiology of bacteriophage and vaccinia virus. Added as an Appendix to the thesis is a group of experiments which have not yet been prepared for publication, but which have a direct bearing on the results discussed.

The various aspects of ultraviolet radiation will be considered in the Introduction under the three arbitrary headings - physico-chemical, biological and genetic.

I. PHYSICO-CHEMICAL PROPERTIES.

Ultraviolet light is an electromagnetic radiation with a wavelength spread from 10 to 4000 A°. The lower limits of the ultraviolet (UV) range overlap with x-rays whose wavelengths range from 10 to 0.1 A°, while the upper limit of the UV radiation, about 4000 A°, is close to the shortest wavelength which can be recognised by the eye as visible light.

For technical reasons the wavelength most commonly used in studies of the effects of UV radiation in biological systems is 2537 A°, about the middle of the wavelength band. The energy contained in a quantum of such radiation can be expressed by the quantum equation, $E = h\nu$.

E is the energy, in electron volts;

h is Planck's constant (4.14×10^{-15} electron volt seconds);

ν is the frequency of the radiation.

The frequency of the radiation, ν, is related to the wavelength by the expression, $\nu = \frac{c}{\lambda}$, c being the velocity of light (3×10^{10} cm/sec.), and λ, the wavelength under examination. The energy in one quantum of ultraviolet light of 2537 A° wavelength therefore is $\frac{hc}{\lambda}$.

$$E = 4.14 \times 10^{-15} \times 3 \times 10^{10} \;/\; 2537 \times 10^{-8}$$

electron volts;

= 4.96 electron volts.

This quantal energy can be transferred to a molecule through absorption by one of its electrons. The absorption of quanta of radiant energy by molecules is determined by their internal structure. In a particular molecule electrons can exist only in a discrete number of energy states, and a definite amount of energy is required to raise an electron from one state to another. As an electron cannot absorb a fraction of a quantum, that is, it must absorb all or none at all, it is evident that for absorption to occur, the amount of energy within a quantum must be exactly that by which an electron may be raised to a higher state. The process whereby an electron is raised from one energy state to another through the absorption of a quantum of radiant energy is known as excitation. The energy required for excitation varies with the molecule concerned but is in the region of 3 to 20 electron volts. Ultraviolet light, with an energy of approximately 5 electron volts, is therefore able to produce excitation, but ejection of an electron from an atom, ionisation, cannot be caused by this source of radiation as the energy required for that process is from 10 to 30 electron volts.

<u>Absorption Spectra</u>. The dependence of absorption of UV radiation upon molecular structure is used as an aid in the identification of the material under study. When optical density is plotted against the wavelength, the resultant curve is called an absorption spectrum. Each class of molecules has a characteristic absorption spectrum, and if certain criteria of purity, homogeneity, and absence of light scattering are met, it is possible to determine the main components of the system being studied. The absorption spectra of nucleic acids show a characteristic peak around 2600 Å, this peak being due to the purine and pyrimidine rings within the nucleic acids. Proteins which contain the aromatic amino acids also show some absorption in the region 2400 to 3000 Å but the major absorption peaks for proteins are below 2400 Å.

If the extinction coefficients of proteins and nucleic acids are compared at 2537 Å, it is found that the extinction coefficient per unit weight of nucleic acid is thirty times greater than that of protein, that is, about 30 times more radiant energy will be absorbed per unit weight of nucleic acid than protein when the two are irradiated as mixtures.

In essence virus particles are mixtures of protein and nucleic acids, the protein acting as a coat for the

nucleic acid. Knowing the extinction coefficients for these substances at the wavelengths used, the size, composition, and concentration of the virus suspension, one can calculate the relative amounts of absorption of UV light by the two major components of the virus. Vaccinia virus contains 5 to 6% deoxyribonucleic acid (DNA), the remainder of the particle being mainly protein. Therefore, although the nucleic acid has a greater absorption capacity than the protein, the composition and size of the vaccinia virus particle would indicate that only 36% of the energy is capable of being absorbed by the nucleic acid. This is in direct contrast to another DNA-containing group of viruses, the T-even bacteriophages, which contain 40% DNA. There, more than 95% of the incident energy is capable of being absorbed by the nucleic acid.

Calculated and observed absorption measurements are, however, not always in agreement. With animal viruses it has been extremely difficult until recent years to meet the necessary criteria. Purity and homogeneity have been the main problems, for concentrated virus preparations inevitably contain considerable amounts of host-tissue components, and the absorption spectra obtained were nearer those of the host-tissue than the

virus. Even where satisfactory virus preparations have been used, the interpretation of the absorption spectra has difficulties of its own. The spectrum obtained from the virus preparation may differ from the expected because of the form which the nucleic acid takes up inside the protein coat. For example, with T6 and Shope papilloma virus (Bonhoeffer and Schachman, 1960) the DNA is coiled so tightly within the virus that absorption spectrum studies would indicate only 70% of the true amount of DNA present, if the hypochromic effect would not be taken into account. The scattering of light by the virus particles can also lead to misinterpretation of absorption spectra.

The preceding discussion should indicate that the conclusions drawn from the published accounts of absorption spectra of many viruses were often not based on the stricter scientific criteria acceptable today. Their value is therefore largely historical. Viruses whose absorption spectra fall into this class include tobacco mosaic virus (Bawden and Pirie, 1937), tomato bushy stunt virus (Bawden and Pirie, 1938), Eastern equine encephalomyelitis virus (Taylor et al., 1941, influenza (Hollaender and Oliphant, 1944), and bacteriophage T2 (Dulbecco, 1950).

II. BIOLOGICAL EFFECTS OF UV.

Inactivation Kinetics. When virus particles are irradiated with UV light, the first characteristic of the virus to be damaged by the radiation is the ability to infect cells, and replicate therein. However, as infectivity is, for most viruses, the major characteristic studied after UV radiation, it is possible that other properties of which we have no cognisance at present, may be more readily inactivated than infectivity. Kleczkowski (1957) has reported that, with TMV irradiated with UV, a delay in multiplication, a decrease in stability, a loss of ability to multiply when the host is kept in darkness, and an increased frequency of genetic mutation occur before the total inactivation of the virus. A study (Henle and Henle, 1947) of the inactivation of influenza virus by UV showed that the known properties of the virus were inactivated in the following order: infectivity, toxicity, immunizing capacity, and finally, the ability to agglutinate red blood cells.

It is commonly assumed that viruses are inactivated by UV radiation according to first order kinetics, that is that the rate of inactivation follows the exponential equation, $\frac{V}{V_o} = e^{-kD}$ where

V_o is the titre of the unirradiated sample,

V is the titre of the irradiated sample,

D is the dose of radiation,

k is a constant, characteristic for a given virus and the conditions of irradiation.

Inactivation curves are obtained by plotting the logarithm of $\frac{V}{V_o}$ against the dose which, being delivered at a constant rate, can be measured in units of time. This would imply that the dose of radiation was the only relevant variable in virus inactivation. However, improved techniques in recent years have given the virologist more precise methods for titrating his material, and it would now appear that deviations from the logarithmic rate of inactivation are frequent. (Dulbecco, 1950; Benzer, 1952; Baron et al., 1959). The inactivation curves of the T-even bacteriophages exhibit a shoulder at low UV doses, that is, there is a slow initial rate of inactivation which changes to a logarithmic rate as the dose increases; for the T1 and T7 phages, there is an initial logarithmic rate which breaks to a slower rate, though again logarithmic, at higher doses of radiation. Inactivation curves similar to that of T1 have been obtained for the animal viruses, herpes and vaccinia (Baron et al., 1959).

Quantum Yield. Where the inactivation curve does obey

first order kinetics, it is possible to calculate the quantum yield. If the inactivation equation is expressed in the following form, $\frac{V}{Vo} = {}^{-k_2x}$ where x is an expression for the average number of quanta absorbed by the virus particle, then the constant k_2 becomes known as the quantum yield. That is, if the absorption of a single quantum of UV radiation were sufficient to inactivate a virus particle and every quantum absorbed inactivated, then the value of k_2 would be 1. The efficiency of inactivation of viruses by UV falls very short of this, however, as the quantum yields for the inactivation of bacteriophage and TMV show. For bacteriophage T2 at 2537 Å, the quantum yield is between 3 and 6 x 10^{-4} (Zelle and Hollaender, 1954). That is, to inactivate a T2 particle, an average of 1500 to 3000 quanta is required.

Action Spectrum. If the efficiency of inactivation of a virus particle is plotted against the wavelength used for inactivation, a curve known as the action spectrum is obtained. It will be evident from the discussion on absorption spectra that the peak in the action spectrum will also give an indication of the class of compound which is absorbing the UV light. With most viruses it has been found that the action spectrum bears a relationship to the absorption spectrum of the nucleic

acid within the virus (Rivers and Gates, 1928; Zelle and Hollaender, 1954). Although these results seem to support the belief that the nucleic acid is the essential absorbing molecule, caution must again be exercised in interpretation of the results obtained.

<u>Role of Nucleic Acids</u>. Better confirmatory evidence for the definitive role of nucleic acids in radio-biological reactions are the experiments on the rate of change of inactivation of phage-bacterial complexes as a function of the growth cycle, the Luria-Latarjet experiments (Luria and Latarjet, 1947). The principle of the Luria-Latarjet experiment is that in singly infected bacterial cells, the change in the sensitivity of the complex should reflect only the changes caused by the replication of the phage. As the number of phages increases, the shape of the inactivation curves should alter from a single hit to multihit curve, giving a shoulder, the extent of which should increase with the number of particles. Survival curves similar to the theoretical ones were obtained with T7, but in the case of the T-even phages (Benzer, 1952) the predicted shoulder did not arise. A discussion of these results may be found in Stent (1958) and Stahl (1959). However, the survival curves for the first two or three minutes with both groups of phage, are similar to those of free

phage. As it is known that only the DNA and 3% of the total protein of the phage enter the bacterial cell to initiate replication, it seems certain that the DNA is the primary absorbing moiety.

The most definitive evidence for the role of nucleic acids is the inactivation by UV of the infective nucleic acid which has been extracted from the protein coat of the virus. For example, treatment of infective RNA from Tobacco mosaic virus results in an inactivation of the ability of the nucleic acid to produce lesions on the plant leaf (Siegel et al., 1956). Similar results have been obtained with poliovirus nucleic acid (Norman, 1960).

In summary of this section it may be said that the primary biological effect of UV radiation is an excitation process which destroys the functional capacity of the absorbing molecule. It is generally believed that the nucleic acid component of the virus is the primary absorbing molecule.

<u>Inactivation Studies</u>. As would be expected from the foregoing discussion, most of the papers published on the effects of UV on viruses are concerned with the inactivation process per se. The first available report

is one by Friedberger (1914) who used UV to obtain a suspension of vaccinia virus free from contaminating bacteria, a very common contingency of the pre-antibiotic era. This he was able to do, and still maintain the infectivity of the vaccinia virus. For at least another 20 years, UV was used for inactivation or absorption spectra studies.

The opening up of the bacteriophage field by Twort in 1915 resulted in many of the early UV studies being made with bacteriophage (McKinley et al., 1926; Fisher and McKinley, 1928; Gates, 1934). As there were few methods for satisfactory titration of the available animal viruses, the range of viruses studied was limited. Among those which were investigated, mainly on a qualitative rather than a quantitative basis, were vaccinia (Carnot et al., 1926; Rivers and Gates, 1928); vaccinia and herpes (McKinley et al., 1926); Rous sarcoma virus (Baker and Peacock, 1926); tobacco mosaic virus, TMV (Stanley, 1936); and poliovirus (Toomey, 1937). Once satisfactory methods for titrating virus became established - the use of the embryonated hen's egg (Burnet, 1936) and in particular, the plaque technique on tissue cultures (Dulbecco, 1952), more accurate studies could be made of the inactivation of viruses by UV. Henle and Henle (1947), studying

influenza virus, Collier et al. (1955), studying vaccinia, Dulbecco and Vogt (1955), studying poliovirus, and Rubin and Temin (1959), studying Rous sarcoma virus and Newcastle disease virus, are all examples of the more detailed studies which have been made possible by increasing the accuracy of the methods of assay.

Photoreactivation. Inactivation studies will undoubtedly result in research into reactivation processes. Photoreactivation, whereby visible light is able to repair a proportion of the damages by UV and so lower the inactivating effect of the radiation, was found to occur with bacteriophages by Dulbecco (1950). It is significant that with bacteriophage, the phenomenon can only occur when the phage-bacterium complex is illuminated - no photoreactivation can be demonstrated with extracellularly illuminated phage. Although many strains of bacteriophage have been shown to undergo photoreactivation (Kleczkowski and Kleczkowski, 1953; Dulbecco, 1955), there is no available report of photoreactivation of an UV-irradiated animal virus. As with bacteriophage, Bawden and Kleczkowski (1952) found photoreactivation of tobacco necrosis virus and tomato bushy stunt virus only when the virus-leaf complexes were illuminated. With TMV, photoreactivation could be shown only with the nucleic acid-leaf complex (Bawden and Kleczkowski, 1959) - the

intact virus-leaf complex gave consistently negative results.

Although Bowen (1953) has elucidated some of the steps in photoreactivation, the mechanism is still unknown. Experiments by Lennox et al. (1954) indicate that the reactivation is the result of a direct repair of the UV damage.

III. GENETIC EFFECTS OF UV.

The demonstration by Delbrück and Bailey (1946) of a genetic interchange between different strains of T4 bacteriophage opened the vast field of bacteriophage genetics in which irradiation has proven an illuminating tool. From the careful detailed studies which have been made on the effects of UV on such genetic phenomena as mutation frequency, genetic recombination, marker rescue and multiplicity reactivation, much valuable information has been obtained on the complex problem of bacteriophage replication.

Mutation Frequency. There have been reports of an increase in the frequency of mutations among survivors of irradiated phage-bacterial complexes (Latarjet, 1949) and of T4 phage irradiated extracellularly and used for multiple infection of the bacterial cell (Krieg, 1959b). The significance of multiplicity reactivation in these cases has yet to be determined. In those bacteriophages

where there is a partial homology between the phage and bacterial genomes, the reports of mutation with irradiated virus-host complexes (Weigle and Dulbecco, 1953; Weigle, 1953); with phage inoculated into irradiated host cells (Jacob, 1954; Fraser, 1957), or with irradiation of phage alone (Tessman, 1956), must be interpreted with great caution.

Genetic Recombination. An increase in recombination in crosses with phage λ was found by Jacob and Wollman (1955) when one, or both, of the parent strains were irradiated with UV. A similar effect has been found for the T-even phages (Streisinger and Franklin, 1956; Epstein, 1956).

Cross Reactivation. Cross reactivation involves the rescue of a marker from a non-viable parent by another related viable parent phage. An extensive study, particularly by the Rochester group (Doermann et al., 1955; Krieg, 1959a), of the phenomenon of cross reactivation has resulted in a considerable amount of information which has played an important role in the proposed theories of bacteriophage replication (Stent, 1958; Krieg, 1959a; Stahl, 1959). These experiments will be discussed in more detail in the General Discussion.

Multiplicity Reactivation. Multiplicity reactivation is the phenomenon whereby irradiated virus particles which, individually are unable to replicate, can cooperate in

multiple infection to produce viable progeny. This phenomenon was discovered by Luria (1947) with various T-even phages, and investigated in more detail by Luria and Dulbecco (1949); Dulbecco (1952); Harm (1956); Barricelli (1956, 1960), and Epstein (1958). Multiplicity reactivation has been shown to occur with irradiated influenza virus (Henle and Liu, 1951; Barry, 1960), and perhaps with poliovirus (Drake, 1958). Multiplicity reactivation, like cross reactivation, is very difficult to demonstrate with those phages possibly having an homology between the phage and bacterial genomes.

These genetic aspects of the effects of UV on viruses will be discussed more fully in the General Discussion where a comparison of the radiobiology of bacteriophage and vaccinia will be made. It is pertinent here to acknowledge the great debt which animal virologists working on radiation phenomena owe to the bacteriophage workers. As Stahl (1959) stated in his review of the radiobiology of bacteriophage, "It is equally likely that the experiments and ideas applied to T-even radiobiology will supply guidance for future studies with other systems."

In conclusion, as a Preface to the experimental data to be reported, an extract is given from one of the

first systematic studies on the properties of light, Huygens' "Treatise on Light" (1690). This extract, although 270 years old, is a perfect expression of the thoughts of a 20th century radiobiologist.

"There will be seen.....demonstrations of those kinds which do not produce as great a certitude as those of Geometry, and which even differ much therefrom, since whereas the Geometers prove their Propositions by fixed and incontestable Principles, here the Principles are verified by the conclusions to be drawn from them; the nature of these things not allowing of this being done otherwise. It is always possible to attain thereby to a degree of probability which very often is scarcely less than complete proof. To wit when things which have been demonstrated by the Principles that have been assumed correspond perfectly to the phenomena which experiment has brought under observation; especially when there are a great number of them, and further, principally, when one can imagine and foresee new phenomena which ought to follow from the hypotheses which one employs, and when one finds that therein the fact corresponds to our prevision. But if all these proofs of probability are met with in that which I propose to discuss, as it seems to me they are, this ought to be a very strong

confirmation of the success of my inquiry; and it must be ill if the facts are not pretty much as I represent them. I would believe then that those who love to know the causes of things and who are able to admire the marvels of Light, will find some satisfaction in these various speculations regarding it ....I hope also that there will be some who by following these beginnings will penetrate much further into this question than I have been able to do, since the subject must be far from being exhausted. This appears from the passages which I have indicated where I leave certain difficulties without having resolved them, and still more from matters which I have not touched at all........Finally, there remains much more to be investigated touching the nature of Light which I do not pretend to have disclosed, and I shall owe much in return to him who shall be able to supplement that which is here lacking to me in knowledge."

Huygens (1690)

ORIGINAL INVESTIGATIONS PRESENTED AS PAPERS
PREPARED FOR PUBLICATION

Paper I.

The effects of Ultraviolet Radiation on Vaccinia Virus. 1. Inactivation, Mutation, and Marker Rescue.

Paper II.

Topography in Vaccinia Virus Genetics.

Paper III.

The Effects of Ultraviolet Radiation on Vaccinia Virus. 2. Multiplicity Reactivation.

THE EFFECTS OF ULTRAVIOLET RADIATION ON VACCINIA VIRUS:

1. INACTIVATION, MUTATION AND MARKER RESCUE.

by

Pamela Abel

Department of Microbiology,
John Curtin School of Medical Research,
Australian National University,
Canberra, Australia.

SUMMARY

Ultraviolet irradiation of vaccinia virus results in an inactivation curve having three exponential components. At high UV doses there is a marked increase in the proportion of white u mutants among survivors of irradiated RP u^+. The results can be interpreted as mutations induced by UV and expressed through multiplicity reactivation. The rescue of the u^+ marker from irradiated wild type RP u^+ by the mutants u1 and u2 has also been demonstrated.

INTRODUCTION

Ultraviolet radiation has served well in the understanding of bacteriophage replication (Stent, 1958). Its possibilities have been much less exploited in the field of animal virology, mainly for lack of a suitable system combining means for quantitative study with occurrence of genetic interaction.

The second demand is filled by the group of pox viruses, where genetic interaction has been shown (Fenner and Comben, 1958; Gemmell and Cairns, 1959), and readily distinguishable stable pock mutants are available for detailed study. To meet the first demand, improved quantitative techniques were developed for the assay of free virus and of infective centers, and will be described below. With a combination of these techniques, the radiobiology of vaccinia virus was studied; the first part of these investigations will report on the kinetics of inactivation, the effect of irradiated

virus on its host, and on the rescue of genetic markers from the irradiated virus.

ABBREVIATIONS.

CAM: chorioallantoic membrane; CEF: chick embryo fibroblasts; UV: ultraviolet radiation; BSS: balanced salts solution.

MATERIALS AND METHODS

Viruses

Vaccinia-Mill Hill (VMH), and Rabbit Pox - Utrecht ($RP\underline{u}^+$), both of which have been described by Fenner (1958, 1959) while for the genetic work two mutants of $RP\underline{u}^+$, u1 and u2 (Gemmell and Cairns, 1959) were used. All preparations were fluorocarbon extracts (Gessler et al., 1956) of infected CAMs showing confluent lesions.

CAM titrations

Suspensions of virus were assayed by the method of Westwood et al., (1957), in 11 or 12 day eggs.

Cell cultures

Chick embryo fibroblasts were prepared from decapitated 10 or 11 day chick embryos by the method of Franklin et al., (1957). They were grown in a medium containing 8% calf serum, 0.25% lactalbumin hydrolysate in Hanks' balanced salts solution (Hanks

and Wallace, 1949). Suspensions of the fibroblasts were plated in 5 ml volumes in 5 cm diameter Petri dishes, and incubated in a gassed, humidified incubator at 37°C. Monolayers formed overnight. Before use they were washed once with Hanks' BSS, and the virus inoculated in 0.1 ml volumes. After a 4 hrs adsorption period, they were overlaid according to Franklin et al., (1957); however with the concentration of agar reduced to 0.7%.

Alternatively, 5 ml suspensions of fibroblasts were directly inoculated in Petri dishes. After monolayers had formed overnight the medium was removed and the plates overlaid as above.

Both "monolayer plates" and "primary suspension plates" were stained with neutral red 5 days after infection.

KB cells (Eagle, 1955) were grown for 12-18 hrs in a medium of 15% human serum and 0.5% lactalbumin hydrolysate in Hanks' BSS.

The cells were dispersed with a mixture of 0.01% trypsin and 0.04% ethylene-diamino-tetra-acetate (EDTA) in Puck's saline (Marcus et al., 1956). For use in the infective center assay the cells were transferred into centrifuge tubes, washed twice in Hanks' BSS, containing 0.5% calf serum, and resuspended in 1 ml of the same medium. All preparations of cells were counted in a haemocytometer before the experiments.

Ultraviolet irradiation

The source of radiation was a Philips 15 watt low pressure mercury vapour lamp emitting more than 95% of its energy at 2537 Å. Its rated output was 750 ergs/cm^2/sec. Samples were exposed in open 5 cm diameter Petri dishes, 60 cm from the source. Gelatin saline (Fazekas de St.Groth et al., 1958) was used as suspending medium and, to ensure uniform exposure of the sample, the suspension was stirred by a megnetic stirrer through the entire period of the irradiation. Under the conditions of irradiation and assay, no photoreactivation took place. The decrease in infectivity was followed by titrating 0.1 ml aliquots of the irradiated suspension on CEF by the primary suspension method.

Following the principles of biological control proposed by Marcus and Puck (1958), a suspension of bacteriophage T1 was introduced into each sample before irradiation, and irradiated aliquots were assayed for phage by the soft agar layer technique (Adams, 1959).

EXPERIMENTAL RESULTS

I. Development of infective center technique, and comparison of assay methods.

The nature of the work to be reported demanded a technique by which the fraction of virus-yielding cells within a population could be estimated. To this end the infective center assay

developed in the phage field (Ellis and Delbruck, 1939) was adapted to vaccinia-infected cells.

KB cells were infected in suspension (about 10^6 cells in 0.2 ml of Hanks' BSS containing 0.5% calf serum) by adding 0.1 ml of the virus preparation. Half an hour at room temperature was allowed for the adsorption; the cell suspension being magnetically stirred throughout the adsorption period by a small teflon-covered stirrer. At the end of the adsorption period, the cells were washed twice with 5 ml of medium, and finally resuspended in 1 ml of KB growth medium. During the growth period the cells were maintained at 37°C in glass-stoppered siliconed tubes, under continuous magnetic stirring.

The infective center assays were always done on 3 hr-incubated cultures, by removing an aliquot from the growth tube, appropriately diluting it, and adding 0.1 ml to 5 ml of CEF suspension as described under Materials and Methods.

During the development of the technique, it was found that treatment with antibody after the adsorption period lowered only negligibly the final titers, that is, residual extracellular virus was not present in significant quantities. Further, the titers were essentially the same whether the cells were plated at 0, 3, or 6 hrs after adsorption. Of the three host systems used for the assay of infective centers, the CAM is of lowest sensitivity and highest variability; CEF monolayers give more

TABLE I

Infective Center Assay of KB Cells Infected in Suspension with Rabbitpox Virus [a]

Method of assay [b]	Use of antibody [c]	Time of assay 0	3	6 hours
CAM	+	50.4 [d,e]	40.6	54.0
	-	63.2	33.0	62.2
Monolayer of CEF	+	48.0	48.8	56.3
	-	58.5	56.8	60.5
Primary suspension of CEF	+	71.6	69.0	72.4
	-	77.6	81.8	80.6

a : $10^{6.3}$ KB cells were infected in suspension with $10^{7.0}$ pock forming units of rabbit-pox virus. After 30 mins adsorption at room temperature, the cells were washed twice, then resuspended in KB growth medium at 10^5 cells/ml, and kept in suspension at 37°C.

b : Details of methods of assay are found under Materials and Methods.

c : At the various times after infection, 10^5 cells were removed from the growth tube, centrifuged down, and resuspended in either 0.5% calf serum in Hanks' BSS or 0.5% calf serum in Hanks' BSS + 10% antivaccinial rabbit serum for 30 mins at 37°C. The cells were then spun down, and diluted out ready for assay.

d : Mean count of 6 eggs or plates.

e : Approximately 82 cells would be in each 0.1 ml inoculum.

reproducible results, but are significantly less sensitive than the assay using the primary suspension method with CEF. The results from this set of experiments on $RP\underline{u}^{+}$ virus are given in Table 1.

(Table 1)

The main conclusion from the experiment is that in a system, where 32 hrs after infection, 88% of the cells (417 out of 473) show fluorescence when stained with fluorescent anti-vaccinia serum according to Coons and Kaplan (1950), 86.7% scored as infective centers when plated on primary CEF plates.

The classical method (Woodruff and Goodpasture, 1931) of chorioallanotic inoculation is still the most sensitive assay of free virus for members of the pox group; however, the variance of the tests is very high (see e.g., Armitage, 1957). Tissue culture techniques, using direct plating on monolayers, are on the whole much more reproducible, but their sensitivity is usually an order of magnitude below that of pock counts on the CAM. The reproducibility of the orthodox technique was compared with that of primary fibroblast plates. 30 replicates of the appropriate virus suspensions were plated and the mean and variance computed from the results.

(Table 2)

TABLE II

Infectivity Assays of Rabbitpox (RP $\underline{u}^{+}$) Virus

Method of assay [a]	Dilution	Mean count [b]	Variance
CAM	10^{-7}	10.4	47.1
Monolayer of CEF	10^{-6}	36.6	43.7
Primary suspension of CEF	10^{-6}	69.3	74.3

a : Details of methods of assay are described under Materials and Methods.

b : Each count is the mean of 30 eggs or plates.

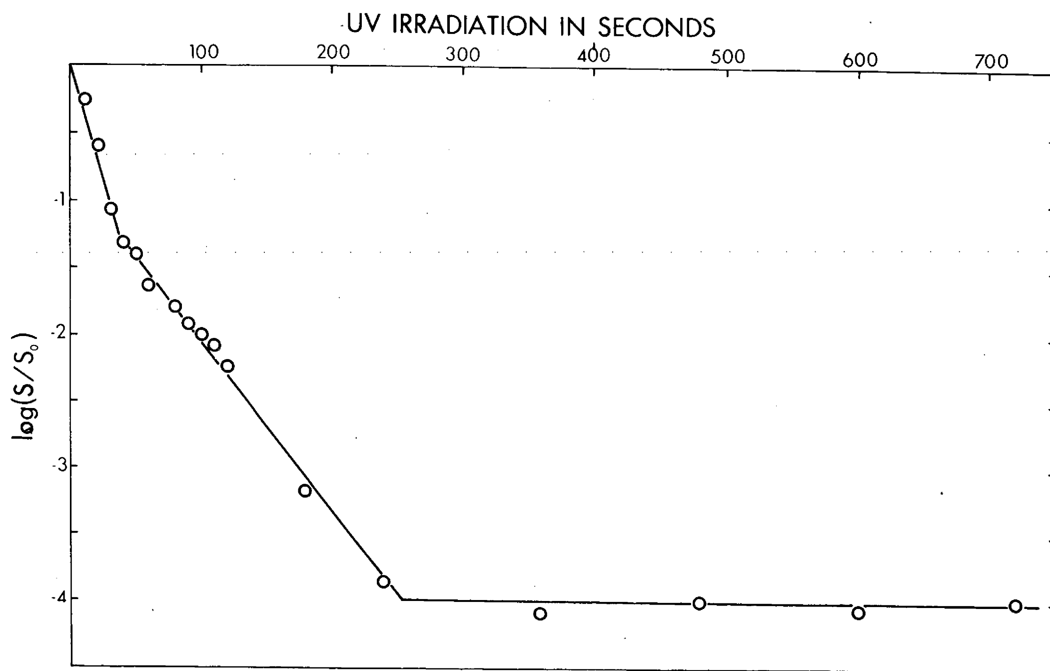
Whereas the titration on the CAM exceeds the other two tests in sensitivity, its variance is almost five times as high as the mean, contradicting a Poissonian model of dose-response relationship. Of the tissue culture methods, the primary suspension is about twice as sensitive to rabbitpox virus than monolayers of the same cells. With other strains of pox viruses the difference in favour of primary cultures is even more striking. Both of these techniques are characterised by variances only slightly in excess of the mean counts, thus the simplest model of random distribution of infective particles over cells of uniform susceptibility can be maintained.

II. Kinetics of inactivation by UV.

Rabbit pox virus at a concentration of $10^{8.3}$ pock forming units per ml was exposed to UV radiation as described under Materials and Methods. To arrive at a firm estimate of the initial slope, samples were taken every 10 seconds for the first 2 minutes and less frequently for the next 10 minutes.

(Figure 1)

The survival curve does not follow simple first order kinetics, but may be regarded as the composite of three linear sections. The curve shown in Figure 1 is characteristic for all pox viruses tested. The first flattening of the curve could be interpreted as heterogeneity in the virus population. To check whether the UV-sensitivity of the original population varied, single plaque



<u>Figure 1</u> UV inactivation of RP $\underline{u}^{+}$ virus. The samples were assayed on CEF monolayers.

isolates were made at 30, 120 and 360 seconds.irradiation. When stocks grown from these isolates were irradiated under identical conditions to the parent stock, it was found that all inactivation curves coincided, thus ruling out heritable inhomogeneity of the parent population. The final levelling of the curve is reminiscent of Luria's finding with the T-even phages (Luria, 1947), there interpreted as multiplicity reactivation. As will be shown later (Abel, 1961), this is also the case in the vaccinia system.

The slopes of these three segments depend somewhat on the medium in which the virus is suspended during irradiation, but the general shape of the inactivation curves remained the same.

III. Cell-killing effect of irradiated virus

When a virus preparation irradiated to reduce its original infectivity by over ten thousandfold is plated on susceptible cells, a large number of cells show marked cytopathic change within 20 min of coming into contact with the inoculum. The number of cells so affected is greatly in excess of the number of infective units in the inoculum. Indeed, in all five experiments of this kind the number of altered cells was higher than even the original infectivity of the preparation. Whether each physical particle is capable of killing the cell it enters cannot be answered at this stage, mainly for lack of a reliable method of recognizing cell death.

To demonstrate that the cell-killing effect is a property of

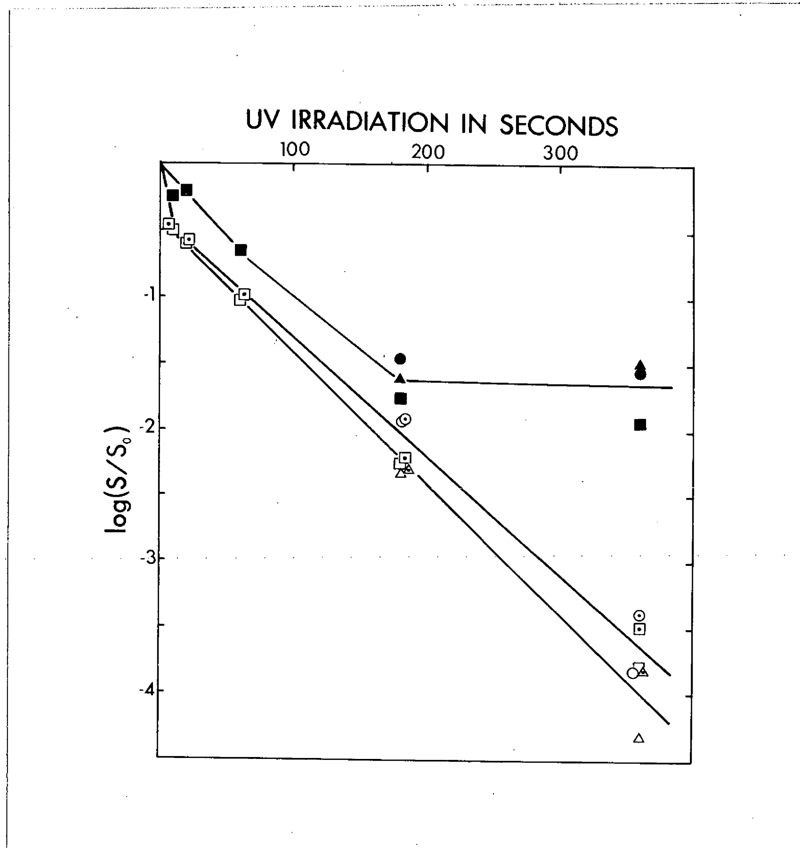
the virus particle, two types of experiment were performed. First, a suspension of virus containing $10^{7.5}$ plaque forming units was irradiated for 12 min., and plated over a sheet of fibroblasts containing $10^{7.5}$ cells. Both this and a ten times smaller dose render the sheet unstainable by neutral red. A hundredfold dilution of the original inoculum gave plates indistinguishable from the uninfected controls. When the original preparation of irradiated virus was centrifuged, the supernate was found to be innocuous, whereas the deposit contained all the activity of the original preparation.

In a second experiment, irradiated virus was treated for 30 min at 37°C with rabbit vaccinial antiserum diluted 1:20. After such treatment no cell-killing activity could be detected.

IV. Change in frequency of mutants on UV-irradiation

Stocks of RP $\underline{u}^+$ contain non-ulcerative mutants ($\underline{u}^-$), with a frequency of about 0.01. (Fenner, 1958). Although phenotypically very similar, these mutations can arise at many points within the genome, and can give rise to the wild type ($\underline{u}^+$) by recombination (Gemmell and Cairns, 1959). If a preparation of the wild type is exposed to UV radiation, the total infectivity will drop as already shown in section II. If the decline of the majority $\underline{u}^+$ and the initially 1% minority $\underline{u}^-$ is plotted separately, the inactivation curves are seen to differ (Fig. 2).

(Figure 2)



<u>Figure 2</u> Differential survival of RP virus on UV radiation. All samples were assayed on the CAM. The three curves represent the inactivation of <u>u</u>$^+$ phenotype (open symbols), of <u>u</u>$^-$ phenotype (closed symbols), and of the total population (dotted symbols). The three separate experiments are represented by circles, triangles and squares, respectively.

The relative increase in the proportion of $\underline{u}^-$ mutants after 3 minutes' irradiation could be interpreted as the outcome of a selective mechanism. To test this possibility, four white mutants were isolated from the population at a survival level of $10^{-3.8}$. By this time the original proportion of white mutants had risen from 1% to 80%, i.e. it is now the majority. Clones derived from the four isolates were irradiated under the same conditions as the original population, that is exposed to the same presumptive selective force. None of the four clones were found to be more resistant to UV inactivation than was the original population. The probability of this finding being due to chance is less than 1/1000, hence a selection hypothesis is rendered unacceptable. Another hypothesis, that of UV-induced mutation, envisages non-lethal as well as lethal damages. Since the number of $\underline{u}$ loci is large (Gemmell and Fenner, 1960), a considerable portion of the non-lethal damages could fall on this part of the genome, and thus induce white mutations at a rate comparable to that of the lethal hits. However, the two kinds of change should be equally observable throughout the course of inactivation. Since this is not the case, the mutation hypothesis cannot hold in its simplest form. As the proportion of white mutants rises at that point where the cells of the assay system are becoming multiply infected, a third hypothesis accounts for the observations by a combination of the mutation hypothesis and presence of multiplicity reactivation. Accordingly, cells infected with more

than one non-viable particle may yield infective progeny. Since the observed excess is almost entirely white, one must postulate that besides the lethal hit most particles must have also sustained hits in one of their many u-loci.

V. Marker rescue from UV irradiated virus

If virus particles rendered non-viable by UV irradiation are used in mixed infections with unirradiated particles, some of the genetic markers of the irradiated parent appear in the progeny. The phenomenon was first demonstrated by Luria (1947) with the T-even phages, and has been extensively analysed by Doermann et al., (1955) and Kreig (1959).

A suitable system for the study of this phenomenon with pox viruses was provided by the finding of Gemmell and Fenner (1960), that two of the u mutants of rabbit pox, u1 and u2, produced very small plaques with a low efficiency of plating, whereas the wild type u^+ gave large plaques.

A mixture of irradiated RP u^+ at an input multiplicity of 0.1 pock forming units per cell, and 50 to 5000 times as much live u1 or u2 was used to infect KB cells. High inputs of the u mutants were used, yet at no time were more than 30% of the KB cells infected by either mutant. The infective centers were plated in CEF. Since the unirradiated parent did not score as infective centers, the plaques appearing were due either to surviving or rescued u^+ phenotype. By assaying in parallel the unirradiated u^+ mixed with u2, a measure of the fraction of u^+

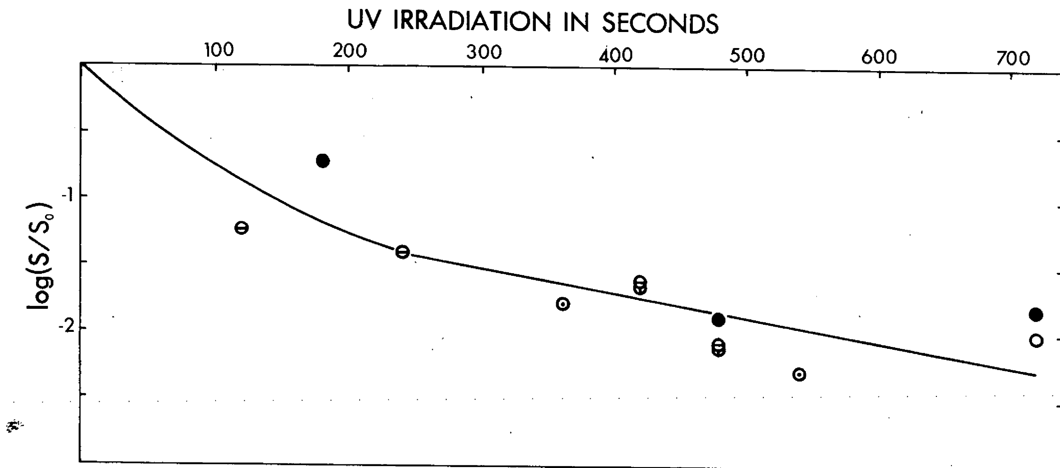


Figure 3 Rescue of $\underline{u}^+$ marker from UV-irradiated RP virus. Mixedly infected KB cells were assayed as infective centers in primary suspensions of CEF.

● rescuing parent RP u1; input multiplicity 40 pock forming units/cell.

⊙ rescuing parent RP u1; input multiplicity 500 pock forming units/cell.

⊖ rescuing parent RP u2; input multiplicity 5 pock forming units/cell.

⦶ rescuing parent RP u2; input multiplicity 50 pock forming units/cell.

○ rescuing parent RP u2; input multiplicity 50 pock forming units/cell.

Irradiated parent RP $\underline{u}^+$; input multiplicity 0.1 pock forming unit/cell.

markers surviving could be obtained as the ratio of the two titers. Figure 3 is a plot of such ratios over the whole of the inactivation curve.

(Figure 3)

The curve resembles those obtained by Doermann et al. (1955). Its steep initial limb is essentially a measure of the inactivation of the $\underline{u}^+$ parent, and the flatter limb of the $\underline{u}^+$ locus.

DISCUSSION

Almost all previous virus radiobiology has been done with bacteriophages, and a comparison of the results obtained with the bacterial viruses and vaccinia, an animal virus, reveals some interesting resemblances.

UV-inactivation of both vaccinia and herpes viruses has previously been shown to result in the same non-linear inactivation curve obtained in this investigation (Baron et al., 1959). Although most of the published UV inactivation curves of poliovirus are linear, (e.g. Dulbecco and Vogt, 1955), Fogh (1955) has obtained an inactivation curve, similar to vaccinia, with one strain of polio, MEF-I. His suggestion that this could be caused by a class of poliovirus with a lower UV sensitivity than the majority of the population was not substantiated by Taylor (1960). A similar conclusion was reached here with vaccinia. With the bacteriophages, T1, T3 and P22, the

concave inactivation curves are believed caused by the phenomenon of partial genetic homology with the host (Garen and Zinder, 1955). The cytoplasmic localisation of vaccinia virus (Cairns, 1960) excludes such an explanation for this virus.

Although the final flattened section of the inactivation curve is not found with the bacteriophages T1, T3 or T7, it is found with T2, T4 and T6 (Luria, 1947). It is significant that the phenomenon of multiplicity reactivation can readily be demonstrated with the T-even phages, but only under certain conditions and with a low efficiency for T1 (Tessman and Ozaki, 1957). In this respect, vaccinia appears to resemble the T-even phages, in that the levelling at high UV doses is caused by multiplicity reactivation. This is discussed more fully in the following paper.

The toxic effect of irradiated vaccinia virus in tissue culture systems has been found by other workers (Nishmi and Berkopf, 1958; Bernkopf et al., 1959; Brown et al., 1959; Hanafusa, 1960). The cytological changes described have varied somewhat with the cell line used but, in all cases, the inactive virus possessed the ability to kill the host cell without concomitant viral replication. This is analogous to the host killing effect of T2 bacteriophage (Luria and Delbruck, 1942). However, Marcus and Puck (1958), who were analysing the cell killing property of NDV in HeLa cells, found that whereas one live virus particle was capable of killing a HeLa S 3 cell, the cell killing capacity was lost when irradiated virus was used.

The increased frequency, at high UV dose, of white pock formers, may be presumed to be due to a mutagenic action of the

radiation, coupled with the expression of such white mutants through multiplicity reactivation. All white mutants tested have shown a sensitivity to UV radiation similar to the wild type virus and the large number of loci at which such mutants might arise, favours such an hypothesis.

Cross reactivation in the animal virus field has been shown for influenza virus (Baron and Jensen, 1955; Gotlieb and Hirst, 1956). However the principal marker studied in both cases was virulence, a complex marker not readily analysed by present techniques. The use of the white mutants of rabbitpox virus enabled a preliminary study to be made of the rescue of the $\underline{u}^{+}$ marker by methods analogous to the bacteriophage T4 system. (Doermann et al., 1955). The vaccinia results again resemble those with the phage in that the dose-response curve would suggest that the probability of marker rescue remains fairly steady at high doses of UV, and that the UV sensitivity of the marker studied is much less than that of the infectivity of the virus.

REFERENCES

ABEL, P. (1961). The effects of ultraviolet radiation on vaccinia virus. 2. Multiplicity reactivation. Virology,

ADAMS, M.H. (1959). Bacteriophages. Interscience. New York.

ARMITAGE, P. (1957). Studies in the variability of pock counts. J. Hyg. 55, 564 - 581.

BARON, S., AND JENSEN, K.E. (1955). Evidence for genetic interaction between non-infectious and infectious influenza A viruses. J. Exptl. Med. 102, 677 - 697.

BARON, S., MILLER, A.H., GOCHENOUR, A.M., AND HIATT, C.W. (1959). Comparative study of ultraviolet light inactivation of animal viruses. Fed. Proc. 18, 557.

BERNKOPF, H., NISHMI, M., AND ROSIN, A. (1959). Effect of active and inactive vaccinia virus preparation of human amnion cell cultures. J. Immunol. 83, 635 - 639.

BROWN, A., MAYYASI, S.A., AND OFFICER, J.E. (1959). The "toxic" activity of vaccinia virus in tissue culture. J. Infect. Dis. 104, 193 - 202.

CAIRNS, J. (1960). The initiation of vaccinia infection. Virology, 11, 603 - 623.

COONS, A.H., AND KAPLAN, M.H. (1950). Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of fluorescent antibody. J. Exptl. Med. 91, 1 - 13.

DOERMANN, A.H., CHASE, M., AND STAHL, F.W. (1955). Genetic recombination and replication in bacteriophage. J. Cellular Comp. Physiol. 45, Suppl. 2, 51 - 74.

DULBECCO, R. AND VOGT, M. (1954). One-step growth curve of Western equine encephalomyelitis virus on chick embryo cells grown in vitro and analysis of virus yields from single cells. J. Exptl. Med. 99, 183 - 199.

DULBECCO, R., AND VOGT, M. (1955). Biological properties of poliomyelitis viruses as studied by the plaque technique. Ann. N.Y. Acad. Sci. 61, 790 - 800.

EAGLE, H. (1955). Propagation in fluid medium of a human epidermoid carcinoma strain KB. Proc. Soc. Exptl. Biol. Med. 89, 362 - 364.

FAZEKAS de ST.GROTH, S., GRAHAM, D.M. AND JACK, I. (1958). The serology of mumps infections. I. A new source of antigen and a simplified complement fixation test. J. Lab. Clin. Med. 51, 883 - 896.

FENNER, F. (1958). The biological characters of several strains of vaccinia, cowpox and rabbitpox viruses. Virology 5, 502 - 529.

FENNER, F. (1959). Genetic studies with mammalian poxviruses. II. Recombination between two strains of vaccinia virus in single HeLa cells. Virology 8, 499 - 507.

FENNER, F., AND COMBEN, B.M. (1958). Genetic studies with mammalian poxviruses. I. Demonstration of recombination between two strains of vaccinia virus. Virology 5, 530 - 548.

FOGH, J. (1955). Ultraviolet light inactivation of poliomyelitis virus. Proc. Soc. Exptl. Biol. Med. 89, 464 - 465.

FRANKLIN, R.M. RUBIN, H., AND DAVIS, C.A. (1957). The production, purification and properties of Newcastle disease virus labeled with radiophosphorus. Virology 3, 96 - 114.

GAREN, A., AND ZINDER, N.D. (1955). Radiological evidence for partial genetic homology between bacteriophage and host bacteria. Virology 1, 347 - 376.

GEMMELL, A., AND CAIRNS, J. (1959). Linkage in the genome of an animal virus. Virology 8, 381 - 383.

GEMMELL, A., AND FENNER, F. (1960). Genetic studies with mammalian poxviruses. III. White (u) mutants of rabbitpox virus. Virology 11, 219 - 235.

GESSLER, A.E., BENDER, C.E., AND PARKINSON, M.C. (1956). A new and rapid method for isolating viruses by selective fluorocarbon deproteinization. Trans. N.Y. Acad. Sci. Ser.II. 18, 701 - 703.

GOTLIEB, T., AND HIRST, G.K. (1956). The experimental production of combination forms of virus. VI. Reactivation of influenza viruses after inactivation with ultraviolet light. Virology 2, 235 - 248.

HANAFUSA, H. (1960). Killing of L - cells by heat- and UV-inactivated vaccinia virus. Biken's J. 3, 191 - 199.

HANKS, J.H., AND WALLACE, R.E. (1949). Relation of oxygen and temperature in the preservation of tissues by refrigeration. Proc. Soc. Exptl. Biol. Med. 71, 196 - 200.

KRIEG, D.R. (1959). A study of gene action in ultra-violet irradiated bacteriophage. Virology 8, 80 - 98.

LURIA, S.E. (1947). Reactivation of irradiated bacteriophage by transfer of self-reproducing units. Proc. Natl. Acad. Sci. U.S. 33, 253 - 264.

LURIA, S.E. and DELBRUCK, M. (1942). Interference between bacterial viruses. II. Interference between inactivated bacterial virus and active virus of the same strain and of a different strain. Arch. Biochem. 1, 207 - 218.

MARCUS, P.I. AND PUCK, T.T. (1958). Host-cell interaction of animal viruses. I. Titration of cell-killing by viruses. Virology 6, 405 - 423.

MARCUS, P.I., CIECIURA, S.J., AND PUCK, T.T. (1956). Clonal growth *in vitro* of epithelial cells from normal human tissues. J. Exptl. Med. 104, 615 - 628.

NISHMI, M. AND BERNKOPF, H. (1958). Toxic effect of vaccinia virus on leucocytes *in vitro*. J. Immunol. 81, 460 - 466.

STENT, G.S. (1958). Mating in the reproduction of bacterial viruses. Advances in Virus Research 5, 95 - 149.

TAYLOR, A.R. (1960). Effects of nonionizing radiations on animal viruses. Ann. N.Y. Acad. Sci. 83, 670 - 683.

TESSMAN, I., AND OZAKI, T. (1957). Multiplicity reactivation of bacteriophage T 1. Virology 4, 315 - 327.

WESTWOOD, J.C.W., PHIPPS, P.H., AND BOULTER, E.A. (1957). The titration of vaccinia virus on the chorioallantoic membrane of the developing chick embryo. J. Hyg. 55, 123 - 139.

WOODRUFF, A.M., AND GOODPASTURE, E.W. (1931). The susceptibility of the chorioallantoic membrane of chick embryos to infection with the fowl-pox virus. Amer. J. Pathol. 7, 209 - 222.

TOPOGRAPHY IN VACCINIA VIRUS GENETICS

Multiplicity reactivation of ultraviolet-irradiated vaccinia virus can be demonstrated consistently in chick fibroblasts but not in KB cells (Abel, to be published). Rabbitpox, the virus used in these experiments, grows equally well in both hosts. The striking difference between these cells, however, is their size: chick embryo fibroblasts have a volume of approximately 1.4×10^{-9} cm^3, while KB cells approximately 1.1×10^{-8} cm^3.

Multiple infection of cells with active vaccinia virus results in the formation of discrete virus factories which merge only late in the growth cycle (1). Since the genetic components of at least two virus particles must interact to bring about multiplicity reactivation, effective cooperation can be expected only if the partners are within working distance. This would have a higher probability in small cells than large ones. Thus, on this hypothesis, multiplicity reactivation should be demonstrable even in KB cells if the irradiated particles could be induced to lodge at sites in close proximity to each other within the cell. This might be achieved by infecting cells with aggregated virus instead of the conventional dispersed suspensions.

Rabbitpox virus was prepared by genetron extraction

Table 1

INFECTIVE CENTRES[a] PRODUCED BY CLUMPED AND DISPERSED VIRUS

UV-Irradiation Dose (seconds)	State of Virus Inoculum		Clumped / Dispersed
	Clumped[b]	Dispersed[c]	
0	100×10^3 [d]	160×10^3	0.62
60	66×10^3	10×10^3	6.60

(a) Infective Centre Assay: KB cells were infected in suspension with RP u+; after adsorption and washing, the infected cells were diluted and plated in suspensions of primary chick embryo fibroblasts.

(b) Virus was clumped after irradiation by treatment with an equal volume of 0.1 M $MgCl_2$ at 37° C for 24 hours.

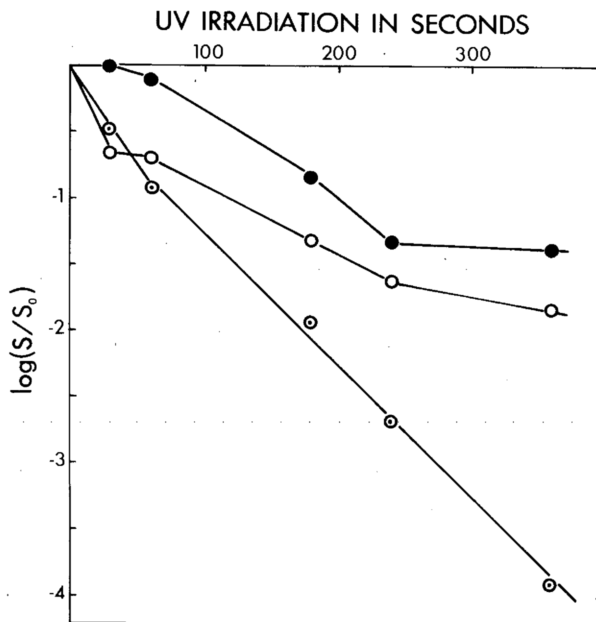
(c) Virus was dispersed by one minute's treatment with an ultrasonic drill.

(d) Plaque forming units per ml.

of infected chorioallantoic membranes (2). Suspensions of virus were exposed in a 2:5 mm layer thickness to a Philips low pressure mercury arc vapour lamp at a rated output of 750 ergs/cm^2/sec., with 95% of the energy emitted at 2537 Å. The suspensions were stirred throughout the period of irradiation and samples withdrawn at various intervals were diluted with an equal volume of 0.1 M $MgCl_2$ (3), and incubated at 37° C for 24 hours. Such preparations of "clumped" virus, as well as "dispersed" virus, obtained from the former by one minute's exposure to a Mullard ultrasonic drill, were used to infect monodispersed KB cells in suspension (Easterbrook, to be published). After adsorption of virus, the KB cells were diluted and 0.1 ml. volumes added to 5 ml. of a suspension of chick embryo fibroblasts. Monolayers formed overnight; the plates were then overlaid with agar and stained with

Table 1 here

neutral red four days later. Table 1 gives the results of such an experiment. As shown in the first line of the Table, clumping of unirradiated virus about halves the number of infective centres, indicating an average clump size of about two infective units. In striking contrast, clumping of UV-inactivated virus raises the number of infective centres by more than six-fold over that of the



<u>Figure 1</u>. Multiplicity reactivation of clumped and dispersed RP virus in KB cells.

- ⊙ UV inactivation curve of free virus, assayed in chick embryo fibroblasts.
- ● Infective centre assay of UV-irradiated clumped virus.
- O Infective centre assay of UV-irradiated dispersed virus.

dispersed virus sample. The overall ten-fold rise can only be due to multiplicity reactivation.

Figure 1

The Figure shows the kinetics of UV-inactivation as tested by clumped and dispersed virus. The inactivation curve of free virus has the same shape as the inactivation curve of dispersed virus when assayed as infective centres in KB cells. The curves coincide over a limited region, and their maximum slopes are at the origin. The inactivation curve of clumped virus in KB cells, however, has a marked shoulder, greatly in excess of what could be predicted from the average clump size, again pointing to multiplicity reactivation.

The demonstration of multiplicity reactivation under conditions of these experiments stresses the importance of topographic factors in vaccinia virus genetics. Further studies on the significance of the phenomenon will be published in detail later.

Pamela Abel

Department of Microbiology,
John Curtin School of Medical Research,
Australian National University,
Canberra. Australia.

1. Cairns, J. *Virology* **11**, 602-623 (1960).

2. Gessler, A.E., Bender, C.E., and Parkinson, M.C. *Trans. N. Y. Acad. Sci.* **18**, 701-704 (1956).

3. Tissieres, A., Watson, J.D., Schlessinger, D., and Hollingworth, B.R. *J. Mol. Biol.* **1**, 221-233 (1959).

THE EFFECTS OF ULTRAVIOLET RADIATION ON VACCINIA VIRUS :

2. MULTIPLICITY REACTIVATION.

by

Pamela Abel

Department of Microbiology,
John Curtin School of Medical Research,
Australian National University,
Canberra, Australia.

SUMMARY

Multiplicity reactivation of ultraviolet-irradiated vaccinia virus can be demonstrated in chick embryo fibroblasts. In KB cells, which have ten times the volume of fibroblasts, the phenomenon can be demonstrated only when the irradiated virus is clumped with $MgCl_2$ before infection of the cells. The consequence of this topographic factor in virus genetics is discussed.

INTRODUCTION

Multiplicity reactivation (MR) is the phenomenon whereby viable virus progeny are produced in cells multiply infected with virus particles which on single infection are inviable. It was first described by Luria (1947) with T-even bacteriophages which had been inactivated by UV.

There have been few studies of MR with animal viruses. Henle and Liu (1951) described multiplicity reactivation with UV-irradiated influenza virus, and their results have recently been confirmed and extended by Barry (1960). Drake (1958) obtained somewhat equivocal evidence of multiplicity reactivation with poliovirus.

This paper describes MR with UV-inactivated vaccinia virus. The importance of the spatial distribution of virus particles within the cell cytoplasm, a phenomenon described briefly elsewhere (Abel, 1961b), is described in more detail in this report.

MATERIALS AND METHODS

These have been described in previous papers (Abel, 1961a and 1961b). Chick embryo fibroblast infective center assays, however, were not used in those papers.

Chick embryo fibroblast (CEF) infective centers

A CEF monolayer was dispersed with 0.125% trypsin; the cells were twice washed in Hanks' BSS containing 0.5% calf serum before counting, diluting and distributing to small siliconed tubes. The infection of the cells, and methods of assay were as for KB cells, except that calf serum medium replaced KB growth medium in the growth tubes.

Multiplicity estimates

In a study of multiplicity reactivation it would seem obligatory that the most important parameter, that of the multiplicity of infection of the cells, should be accurately estimated. With animal viruses however, this is almost impossible to achieve with present techniques. In this paper therefore, no absolute estimates are cited - in almost all experiments relative multiplicities only can be estimated. Although such estimates preclude the evaluation of the efficiency of multiplicity reactivation of vaccinia virus, it is still possible to demonstrate the phenomenon without the knowledge of the actual number of virus particles within each cell.

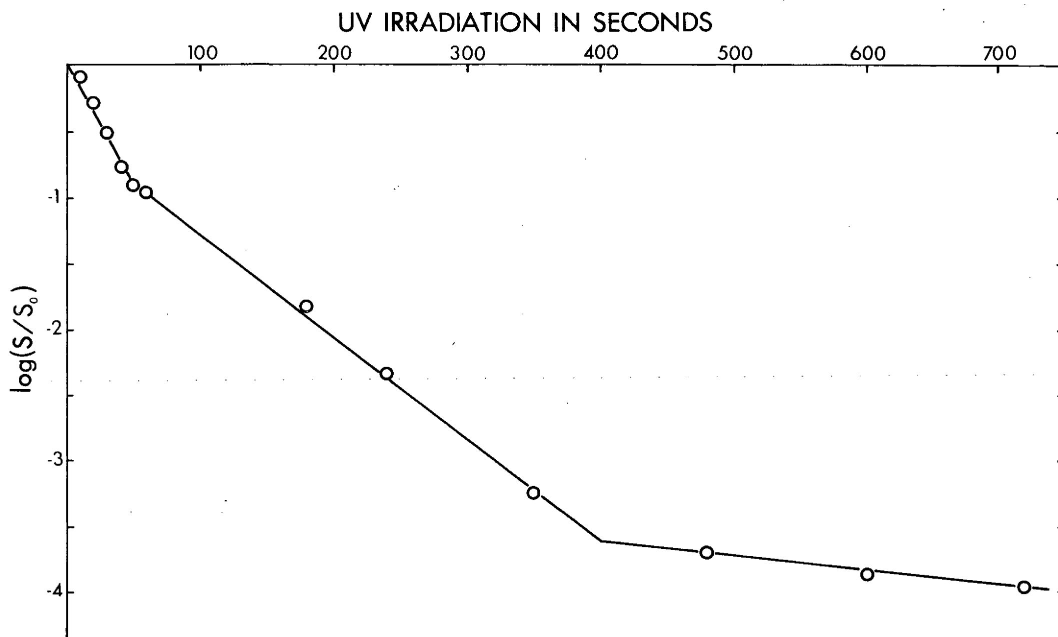


Figure 1 UV inactivation of RP $\underline{u}^{+}$ virus. The samples were assayed on CEF monolayers.

EXPERIMENTAL RESULTS

I. Experiments with chick embryo fibroblasts

When irradiated vaccinia virus was assayed on either the CAM or on CEF, it was noticed that the survival curve flattened out between 6 and 12 minutes' irradiation, after which no surviving virus was detectable, (Figure 1). An analogous result, observed by Luria (1947) in the assay of bacteriophages T2, T4 and T6, inactivated by UV,

(Figure 1)

led to his demonstration of the phenomenon of multiplicity reactivation.

When it had been shown by single plaque isolates that the flattening out with vaccinia was not caused by a genetically resistant fraction of the population (Abel, 1961a), the phenomenon was further studied by altering the multiplicities of infection of fibroblasts. A geometrical progression of infection was achieved in two ways.

1. RP $\underline{u}^+$ irradiated for 0, 420 and 480 seconds was inoculated onto CEF monolayers in doubling volumes, 0.1 ml and 0.2 ml. The results are given in Table 1 from which it can be seen that whereas with the unirradiated virus, the dose-response relationship has a slope of less than one, with irradiated virus, twice the volume yielded 5 to 20 times as many plaques.

(Table I)

TABLE I

The effects of assaying doubling volumes of rabbitpox virus, before and after irradiation.[a]

UV Irradiation (seconds)	Dilution	Volume	Plaque counts[b]
0	10^{-5}[c]	0.05 ml	8
		0.10 ml	15
		0.20 ml	25
420	10^{-1}	0.10 ml	4
		0.20 ml	19
480	10^{-1}	0.10 ml	1
		0.20 ml	20

a RP $\underline{u}^{+}$ was irradiated, diluted, and assayed on chick embryo fibroblast monolayers containing about $10^{7.3}$ cells.

b Each figure is the mean of 6 plates.

c $10^{6.2}$ plaque forming units were equivalent to $10^{7.0}$ pock forming units.

TABLE 2

The effects of assaying doubling dilutions of rabbitpox virus, before and after irradiation.[a]

Experiment	UV Irradiation (seconds)	Dilution	n[b]	Mean plaque counts
I	0	$10^{-5.0}$[c]	6	27
		-5.3	6	15
		-5.6	6	9
		-5.9	6	4
		-6.2	6	2
	480	$10^{0.0}$	6	20
		-0.3	6	4.7
		-0.6	6	2
		-0.9	6	1
		-1.2	6	1
	600	$10^{0.0}$	6	14
		-0.3	6	3
		-0.6	6	4
		-0.9	6	2
		-1.2	6	0

CONTINUED

a RP $\underline{u}^{+}$ was irradiated, diluted and added in 0.1 ml volumes to suspensions of chick embryo fibroblasts containing $10^{6.7}$ cells per plate. The plates were overlaid with agar the next day.

b Number of plates inoculated.

c $10^{6.4}$ plaque forming units were equivalent to $10^{7.0}$ pock forming units.

TABLE 2 (CONTINUED)

The effects of assaying doubling dilutions of rabbitpox virus, before and after irradiation.[a]

Experiment	UV Irradiation (Seconds)	Dilution	n[b]	Mean plaque counts
II	240	$10^{-0.6}$	10	6.5
		-0.9	10	0.1
		-1.2	10	0.0
	360	$10^{-0.3}$	10	16.3
		-0.6	8	4.0
		-0.9	10	0.0
III	180	$10^{-1.3}$	3	19.0
		-1.6	8	3.7
		-1.9	5	1.8

a RP $\underline{u}^{+}$ was irradiated, diluted, and added in 0.1 ml volumes to suspensions of chick embryo fibroblasts containing $10^{6.7}$ cells per plate. The plates were overlaid with agar the next day.

b Number of plates inoculated.

c $10^{6.4}$ plaque forming units were equivalent to $10^{7.0}$ pock forming units.

2. RP $\underline{u}^+$ was irradiated, and a seriesof doubling dilutions made in gelatin saline. These were inoculated in 0.1 ml volumes into primary suspensions of CEF, there being approximately $10^{6.7}$ cells per plate. The results of several experiments recorded in Table 2, again

(Table 2)

show that the dose response relationship of unirradiated virus has a slope of near 1, whereas the slope for irradiated virus is significantly steeper. Similar results were obtained with the V-MH and 7N strains of vaccinia. The inability to express the multiplicity of infection in absolute terms has already been pointed out. Nevertheless, examination of Tables 1 and 2 shows that even estimated multiplicities are far too low to explain the results according to the classical model of multiplicity reactivation. For example, in Table 1, 0.1 ml of unirradiated virus contained $10^{6.2}$ plaque forming units. At 7 and 8 minutes irradiation, the dilution factor is 1 log, that is, 0.1 ml contained $10^{5.2}$ plaque forming units. There are approximately $10^{7.3}$ cells on a CEF monolayer, and therefore even assuming a 100% efficiency of adsorption of the virus, the estimated absolute multiplicity would be only $10^{-2.1}$. At such a multiplicity of infection, the proportion of cells containing two or more plaque forming units would be approximately 0.00014, that is, approximately 1.4 x $10^{3.3}$ CEF would be multiply infected. These values, however, are unreal maximum values. The disproportionate increase in plaque counts on doubling the inoculum

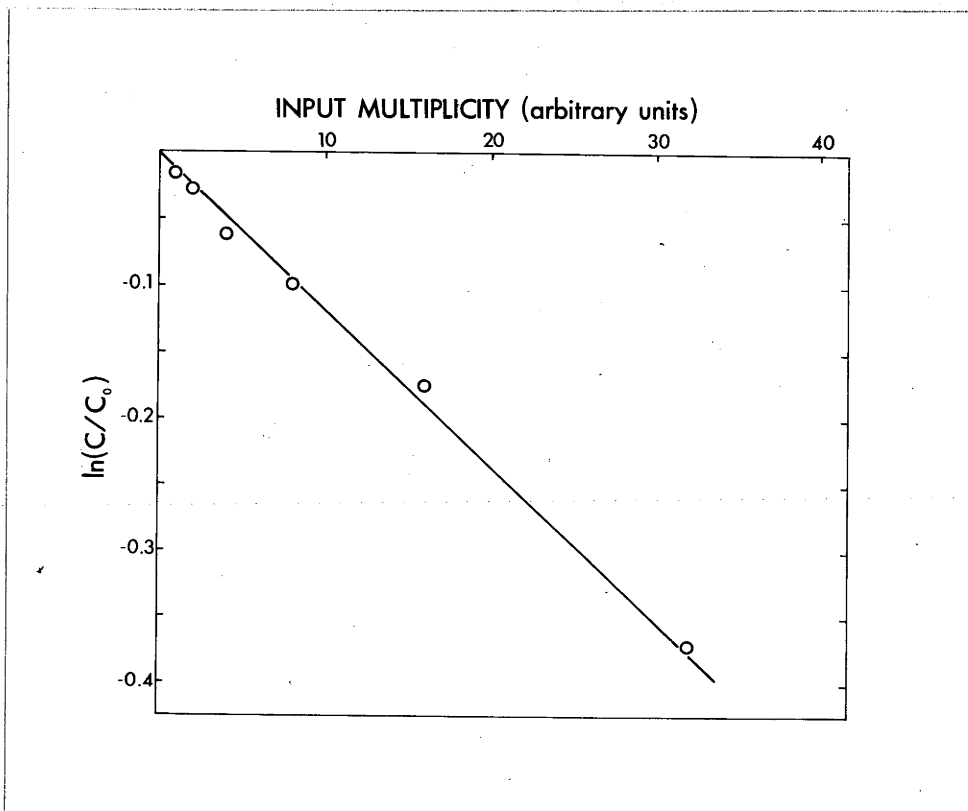


Figure 2 Regression of uninfected cells on input multiplicity. RP virus was used to infect CEF in suspension, and the cells were assayed as infective centres in primary suspensions of CEF. The arbitrary multiplicity of 32 corresponds to an input of 5 pock forming units per cell.

must be due to multiplicity reactivation. Yet relying on the estimates of multiplicity based on pock forming units per cell, and considering the heavy UV dose the particles have sustained, multiplicity reactivation could not occur with the frequency observed. If, however, one takes into account the "non-successful" plaque formers in the virus inoculum - with this stock of RP $\underline{u}^+$, 1 plaque forming unit was equivalent to 7 pock forming units which were equivalent to 70 particles as counted in electron micrographs - the theoretical difficulties are overcome.

As these two types of experiment were obten complicated by the cell-killing effect of irradiated virus (Abel, 1961a), subsequent experiments made use of infective center techniques.

Chick embryo fibroblasts were not considered very satisfactory for infection in suspension because (a) they comprised a non-homogeneous cell population, and (b) preliminary infective center experiments showed a low efficiency of plating. This low efficiency was caused by poor adsorption of the virus to the cells, this being shown when the proportion of fluorescent antibody-stained cells agreed with the proportion of cells registering as infective centers. Figure 2 shows

(Figure 2)

the results of an experiment in which 3.5×10^5 fibroblasts were infected in suspension with 17×10^5 pock forming units, an input

TABLE 3

Multiplicity reactivation in chick embryo fibroblast cells infected in suspension.[a]

UV Irradiation (seconds)	Input Multiplicity (pock forming units/cell)	Mean Plaque Count
0	50	155
	25	83
	12	47
	6	30 [b]
240	50	57.3
	25	15
	12	6
	6	1.3

a $10^{5.7}$ CEF were infected in suspension with RP $\underline{u}^{+}$ irradiated for 0 and 240 seconds, at the multiplicities shown. The infected cells were assayed in primary cultures of chick embryo fibroblasts.

b This anomalous result may be due to an inoculation error.

multiplicity of approximately 5 pock forming units per cell, and with doubling dilutions down to 0.15 pock forming units per cell. At the highest input, more than 90% of KB cells would have become infected, yet only 32% of the fibroblasts registered.

Despite the poor adsorption characteristics of the system, a MR experiment was set up infecting $10^{5.7}$ fibroblasts with RP $\underline{u}^{+}$ irradiated for 0 and 240 seconds. The virus samples were diluted in three two-fold steps and infective centers made with each dilution of the series. The input multiplicities were 50, 25, 12 and 6 pock forming units per cell. The results are shown in Table 3. Even allowing for the inefficiency of the adsorption system, it is obvious that multiplicity reactivation must have occurred.

(Table 3)

II. <u>Experiments with KB cells</u>

In contrast to fibroblasts, KB cells are very satisfactory for infection in suspension (Easterbrook, 1961; Abel, 1961a). However, no evidence of multiplicity reactivation could be obtained with irradiated vaccinia virus and KB cells. Figure 3 shows the results of a typical experiment in which V-MH virus was irradiated and samples taken after 0, 180, 240 and 360 seconds. The samples were diluted in a series of three doubling dilutions which were used to infect $10^{5.6}$

(Figure 3)

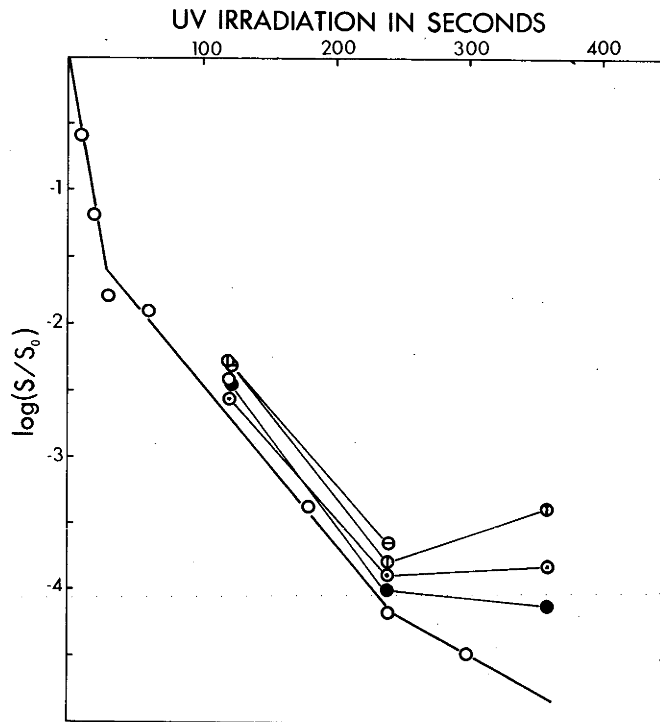


Figure 3 Tests for multiplicity reactivation in KB cells.

○ Inactivation curve of free VMH virus, assayed in primary CEF.

⊙ Infective centre assay of UV-irradiated virus; input multiplicity 6 pock forming units/cell.

⊖ Infective centre assay of UV-irradiated virus; input multiplicity 12 pock forming units/cell.

● Infective centre assay of UV-irradiated virus; input multiplicity 24 pock forming units/cell.

⦶ Infective centre assay of UV-irradiated virus; input multiplicity 48 pock forming units/cell.

KB cells. The input multiplicities were of the order of 48, 24, 12 and 6 pock forming units per cell. In contrast to the results obtained with a similar input in chick embryo fibroblasts, there appears to be no evidence of multiplicity reactivation in KB cells. The virus and infective center survival curves at all multiplicities coincide, except at the final 360 second point. No explanation can be offered for this section of the survival curves.

The divergence of interpretation of the results in fibroblasts and KB cells has been shown to be due to a topographic factor acting in the demonstration of MR in KB cells. (Abel, 1961b). Multiplicity reactivation was shown to occur in KB cells when they were infected with irradiated virus which had been clumped with $MgCl_2$, but not in cells infected with dispersed virus particles. Further results on the demonstration of multiplicity reactivation with clumped virus in KB cells are described in Section III.

III. Experiments with clumped virus

Preparation of clumped virus

0.1M $MgCl_2$ (Tissieres et al., 1959) was found to be a more satisfactory clumping agent than 0.1M $CaCl_2$. The rate of clumping was temperature dependent, the maximum effect being obtained at 37^oC. The following experiment (Table 4) shows that the magnesium ion effect

(Table 4)

TABLE 4

Effect of $MgCl_2$ on Rabbitpox Virus

Diluent[a]	State of Inoculum	Time of treatment in hours			
		0	2	6	24
0.1M $MgCl_2$	Clumped		25[b]	16	5.9
	Dispersed	25	22	23	13
0.1M NaCl	Clumped		23	21	13
	Dispersed	20	24	22	14

a Equal volumes of RP $\underline{u}^+$ and the diluent were mixed together, and incubated at 37°C. At appropriate times samples were removed and titrated immediately in suspensions of chick embryo fibroblasts, and also after treatment for 1 minute with a Mullard ultrasonic drill.

b Plaques x 10^6 per ml. Each figure is the mean of 6 plates.

was that of clumping. Equal volumes of unirradiated virus and 0.1M $MgCl_2$ were mixed and incubated at 37°C. Samples were taken at intervals and assayed immediately, and after treatment for 1 minute with a Mullard ultrasonic drill to break up the clumps. The control samples diluted in normal saline showed that the decrease in titer of the dispersed virus after 24 hours was due to heat inactivation. Treatment with 0.1M $MgCl_2$ regularly caused a drop in titer to less than half of the control figure, and this could be reversed by treatment with the ultrasonic drill. Until electron micrographs can be made of clumped virus preparations, no accurate estimate can be made of the actual clump size.

Multiplicity reactivation as a function of clump size

As the clump size increases, it would be expected that the extent of multiplicity reactivation would rise, both as a function of the clump size, and also of the irradiation dose received by the particles. The former rise would be related to the topography effect, whereas the latter to the phenomenon of multiplicity reactivation itself. To test this prediction, an experiment was set up in which samples of irradiated RP $\underline{u}^+$ were treated with $MgCl_2$ for 2, 6, 12 and 24 hours. Infective centers in KB cells were made from each of these clumped samples, and of free virus as control inoculum. The results

(Figure 4)

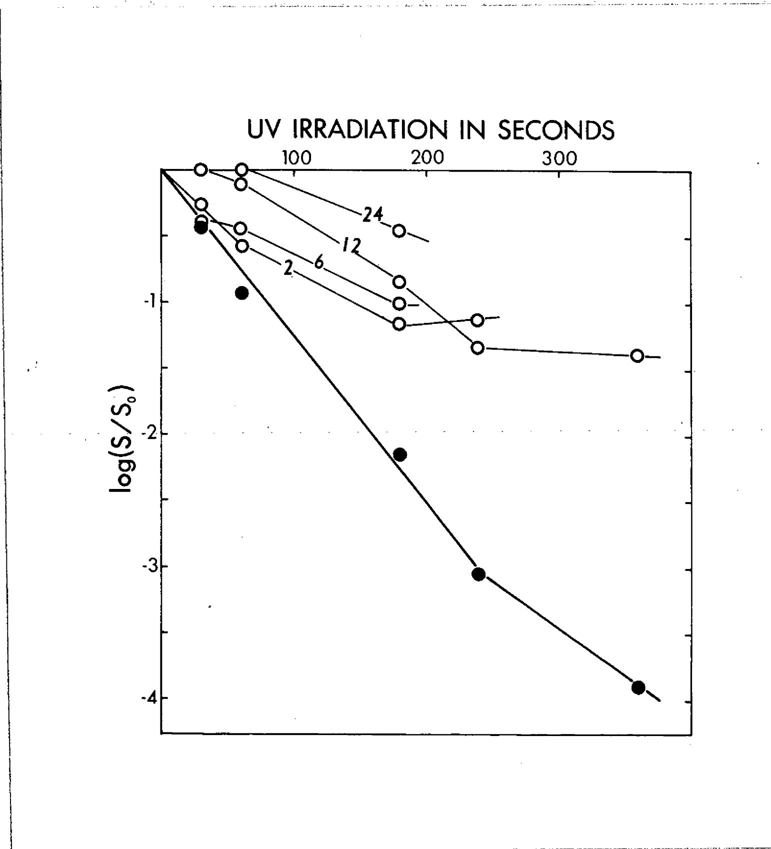


Figure 4 Multiplicity reactivation as a function of clumping time. Free virus (dots) was assayed in primary CEF. Virus, clumped with $MgCl_2$ for the number of hours shown on the curves (circles), was assayed by the KB infective center technique.

are shown in Figure 4. It can be seen that the survival curves of KB cells infected with clumped virus acquire an increasing shoulder as the length of clumping increases. Also, with time of clumping, the deviations of the clumped virus curves from the free virus curve, become more marked.

DISCUSSION

Vaccinia is a DNA-containing animal virus which replicates in the cytoplasm of cells. It is the first of its kind for which genetic interaction has been demonstrated (Fenner and Comben, 1958). After UV irradiation, vaccinia has been shown to undergo multiplicity reactivation, and the study of this genetic phenomenon has added interest in that it revealed the importance of topographic factors in genetic interaction.

That the size of the host cell should have such a pronounced effect on the demonstration of MR was unexpected, yet such a suggestion had been made for MR bacteriophage T4. Cairns and Watson (1956) attempted to show that the deviations from the Luria-Dulbecco (1949) model of MR for bacteriophage T4, as observed by Dulbecco (1952), were caused by a variation in size of the bacterial cells. They believed that the large bacterial cells in the population would adsorb more than the mean number of phage, thus giving a higher probability of the bacterial cell containing a complete set of undamaged units able to

TABLE 5

Relationship between volumes of viral nucleic acids and volumes of recipient cells.

Virus DNA / Host	Volumes in cm^3	Ratio of Volumes
T2 DNA / E. coli	$\frac{7.8 \times 10^{-17}\,[a]}{1.25 \times 10^{-12}\,[b]}$	6.25×10^{-5}
Vaccinia DNA / Chick embryo fibroblast	$\frac{1.54 \times 10^{-16}\,[c]}{1.43 \times 10^{-9}}$	1.08×10^{-7}
Vaccinia DNA / KB cell	$\frac{1.54 \times 10^{-16}}{1.10 \times 10^{-8}}$	1.40×10^{-8}

a Adams, (1959).

b Knaysi (1951).

c Hoagland et al., (1940).

undergo replication. These large cells contributed to the increase in productive complexes, more than was predicted by the Luria-Dulbecco model. The experimental evidence (Harm, 1956) does not bear out the hypothesis. The Cairns-Watson hypothesis is, in practice, the reverse of that found with irradiated vaccinia, where a large cell is incapable of becoming an infective center when multiply infected with irradiated dispersed virus particles. The explanation for this difference lies in the relative volumes of viral DNA to the volume of the recipient cell, as is shown in Table 5. Whereas the phage DNA takes up

(Table 5)

6.25×10^{-5} of the volume of an *E. coli* cell, the DNA of vaccinia takes up only 1.4×10^{-8} of the volume of a KB cell. The probability that a sufficient number of vaccinia particles containing the requisite number of undamaged units would lodge in adjacent sites (Cairns, 1960), is therefore very much lower than for the phage-bacterial system. The effect of topography in the replication of bacteriophages is discussed by Bresch (1959).

The data on MR with vaccinia cannot be analysed by the Luria-Dulbecco model because of the non-linear character of the inactivation curve which precludes an estimate of the hits received. Also, the effect of topography in large cells like KBs, requires a re-assessment of the term, "multiplicity of infection". The estimation of multiplicity

with animal viruses in tissue culture cells is, in itself, a major problem which must be overcome before detailed analytical work can proceed.

The role of the "non-successful" particles, the major fraction of any animal virus preparation, is particularly pertinent in this connection. Presumptive evidence (see Section I) indicates that at least when the overall infectivity of the virus preparation has been reduced by UV, these "non-successful" particles are able to contribute to the production of viable progeny. That is, a fraction of the population behaves as if it already contained something analogous to a radiation damage, and can start replicating only under conditions of some form of multiplicity reactivation. Whether this failure to infect is due to some structural difference, or is the outcome of some statistical factor in the virus-cell interaction, remains to be decided.

Aggregation of virus provides a means by which the topographic barrier in MR can be overcome. More generally, it also provides a new tool in the study of viral genetics since the kinetics of interaction whether in recombination, heat reactivation, cross reactivation or multiplicity reactivation, should depend fundamentally on the state in which the genetic material reaches its host cell and/or its future site of replication.

REFERENCES

ABEL, P. (1961a). The effects of ultraviolet radiation on vaccinia virus. I. Inactivation, mutation and marker rescue. Virology.

ABEL, P. (1961b). Topography in vaccinia virus genetics. Nature.

ADAMS, M.H. (1959). Bacteriophages. Interscience. New York.

BARRY, R.D. (1960). The multiplication of influenza virus. Ph.D. thesis. Australian National University, Canberra.

BRESCH, C. (1959). Recombination in bacteriophage. Ann. Rev. Microbiol. 13, 313 - 334.

CAIRNS, J. (1960). The initiation of vaccinia infection. Virology 11, 603 - 623.

CAIRNS, H.J.F., AND WATSON, G.S. (1956). Multiplicity reactivation in bacteriophage. Nature 177, 131 - 132.

DRAKE, J.W. (1958). Interference and multiplicity reactivation in polioviruses. Virology 6, 244 - 264.

DULBECCO, R. (1952). A critical test of the recombination theory of multiplicity reactivation. J. Bacteriol. 63, 199 - 207.

EASTERBROOK, K.B., (1961). The multiplication of vaccinia virus in suspended KB cells. Virology.

FENNER, F., AND COMBEN, B.M. (1958). Genetic studies with mammalian poxviruses. I. Demonstration of recombination between two strains of vaccinia virus. Virology 5, 530 - 548.

GEMMELL, A., AND CAIRNS, J. (1959). Linkage in the genome of an animal virus. Virology 8, 381 - 383.

GEMMELL, A., AND FENNER, F. (1960). Genetic studies with mammalian poxviruses. III. White (u) mutants of rabbitpox virus. Virology 11, 219 - 235.

HARM, W. (1956). On the mechanism of multiplicity reactivation in bacteriophage. Virology 2, 559 - 564.

HENLE, W., AND LIU, O.C. (1951). Studies on host-virus interactions in the chick embryo - influenza system. VI. Evidence for multiplicity reactivation of inactivated virus. J. Exptl. Med. 94, 305 - 322.

HOAGLAND, C.L., LAVIN, G.I., SMADEL, J.E., AND RIVERS, T.M. (1940). Constituents of elementary bodies of vaccinia. 2. Properties of nucleic acid obtained from vaccine virus. J. Exptl. Med. 72, 139 - 147.

KNAYSI, G. (1951). Elements of Bacterial Cytology, 2nd ed. Comstock (Cornell Univ. Press) Ithaca, New York.

LURIA, S.E. (1947). Reactivation of irradiated bacteriophage by transfer of self-reproducing units. Proc. Natl. Acad. Sci. U.S. 33, 253 - 264.

LURIA, S.E., AND DULBECCO, R. (1949). Genetic recombinations leading to production of active bacteriophage from ultraviolet inactivated bacteriophage particles. Genetics 34, 93 - 125.

TISSIERES, A., WATSON, J.D., SCHLESSINGER, D., AND HOLLINGWORTH, B.R. (1959). Ribonucleoprotein particles from Escherichia coli. J. Mol. Biol. 1, 221 - 233.

GENERAL DISCUSSION

The leading position of the T-even bacteriophages in the field of radiobiology of viruses is the result of two advantages: (a) satisfactory methods of assay, and (b) a satisfactory genetic marker system. Once similar advantages had been obtained for vaccinia virus, it was then possible to attempt a study of the radiobiology of an animal virus.

With bacteriophages T2, T4 and T6 the ratio of plaque forming units to particles visible in electron microscope counts ranged from 0.4 to 1.4 (Luria et al., 1951). The mean efficiency of plating therefore is higher than 0.5, that is, it is possible to assay biologically at least one out of every two actual phage particles. Unfortunately, such a situation does not exist in the animal virus field.

Vaccinia has a rather low efficiency of plating (Dumbell et al., 1957) compared to bacteriophage. With RP $\underline{u}^{+}$, the strain used in most of the experiments described, the efficiency of plating was about 0.1 when the CAM was used as the method of assay. However, the CAM counts had such a high variance that for this study titration in primary chick embryo fibroblasts was used. This method, unfortunately, lowered the efficiency of plating to about 0.014. Nevertheless, the important

considerations of precision and reliability made this method more desirable for this particular study, despite the lower efficiency of plating.

It is unfortunate that RP $\underline{u}^+$ does not have a haemagglutinin, for the haemadsorption plaque assay which was developed for members of the pox group possessing haemagglutinins was both precise and reliable, and more sensitive than the primary assay. This is well demonstrated in the comparative titrations of V-MH virus which are given in the Appendix.

For the cross and multiplicity reactivation experiments it was essential that a satisfactory infective centre technique be available. As with many methods in animal virology, the infective centre techniques were adaptations from the bacteriophage field (Ellis and Delbrück, 1939). However, unlike the phage-bacterial system which is for most purposes standardized (Adams, 1959), in animal virology the methods used in the preparation and assay of infective centres differ markedly depending on the virus strain, the cell strain and the operator. Also, the efficiency of plating of the infected bacterial cell is essentially 1, whereas animal virus-infected cells have efficiencies of plating ranging from 0.04 with a herpes-HeLa system assayed on the CAM (Stoker and Ross, 1958) to 1.0 with herpes-HeLa assayed on HeLa monolayers (Stoker, 1958), and vesicular stomatitis

virus-CEF assayed on CEF monolayers (Franklin, 1958). The system developed for vaccinia virus also arose from practical demands and was conditioned by these; nevertheless it does have a consistent efficiency of plating of 1. The data in the Appendix on the infective centre assays with V-MH virus (Table 5) show similar results to the RP $\underline{u}^+$ experiments described in the first paper. This method would appear applicable to most of the vaccinial strains but its efficiency for other members of the pox virus group has not yet been investigated.

Before the effects of radiation on the genetic properties of an organism can be profitably studied, at least three criteria must be met. These are: (a) a stable genetic marker system which has been adequately characterized; (b) a relatively simple method of scoring the markers, and (c) a satisfactory method of quantitation of both the marker and any radiation-induced change upon it.

An outstanding example of such a set of markers are the $\underline{r}^+$, $\underline{r}$ mutants of bacteriophages T2 and T4 (Hershey, 1946), particularly since the demonstration by Benzer (1955) that the expression of the $\underline{r}$ mutation depended on the host bacterium.

The vaccinia virus group is probably unique at the present time in being the only animal virus group in which

three criteria have been reasonably fulfilled. The major qualification, that of having a stable, adequately characterized marker system, has been worked out by Fenner (1958). In particular, the development of the white mutant system of RP $\underline{u}^+$ (Gemmell and Cairns, 1959; Gemmell and Fenner, 1960) and the fact that two of these mutants, $\underline{u}$ 1 and $\underline{u}$ 2, can be differentiated by plaque production on CEF, has given a satisfactory marker system for cross reactivation studies. Unfortunately, the third characteristic, that of satisfactory quantitation of the genetic effects, returns one again to the relative inadequacies of the vaccinia assay system. The extreme inter-egg variation made studies on the effect of radiation to induce $\underline{u}$ mutants from RP $\underline{u}^+$ rather unsatisfactory, and the poor adsorption of $\underline{u}$ 1 and $\underline{u}$ 2 to KB cells limited the extent of the cross reactivation studies.

The lack of a satisfactory method for the estimation of the multiplicities of infection of vaccinia virus in tissue culture cells remains also a major problem, particularly in the studies on multiplicity reactivation. Nevertheless, the use of relative estimates of multiplicities instead of the preferable absolute estimates, did not prevent the demonstration of the phenomenon.

Despite the difficulties of assay, a beginning was made in the systematic study of the radiobiology of an animal virus, and the results obtained would indicate that as the methods of assay and quantitation are improved, further information should be obtained on the effects of UV radiation on vaccinia virus.

As the statement by Stahl (1959), quoted in the Introduction, that the experiments and ideas applied to T-even radiobiology would supply guidance with other systems, has been borne out with the radiobiological study of vaccinia virus, a comparison of the radiobiology of the two viruses is in place.

For this comparison, the effects of ultraviolet radiation on viruses will be treated in the sequence given in Adams (1959). Where appropriate, the results obtained with other viruses will be compared with the phage results.

The effects of radiation as listed by Adams are: (a) Lethal effect; (b) Growth delaying effect; (c) Host killing; (d) Interference; (e) Photoreactivation, and (f) Multiplicity reactivation. Two further effects to be discussed are: (g) Cross reactivation, and (h) Induction of mutations. As the growth delaying effect (b), and photoreactivation (e) were not studied with vaccinia virus, they will be deleted from this discussion.

(a) Lethal Effect.

Ultraviolet radiation kills both phage and vaccinia. However, the difference in the inactivation curves between the T-even phages and vaccinia virus indicates that the kinetics of inactivation are not identical. Whereas the UV-inactivation curves of the T-even phages show a slight shoulder, those of the vaccinia strains show a concave type curve with three components. This concave type of inactivation curve has been observed for T1, T3, T7, P22 and λ, and has been explained in terms of partial genetic homology between the phage and host, leading to host-mediated reactivation. This explanation is inapplicable to the vaccinia inactivation results, for the cytoplasmic replication centre of vaccinia virus is too remote from the host nuclear DNA for reactivation to occur. It is also unlikely that the concave curves found for herpes (Baron et al., 1959), and poliovirus (Fogh, 1955) are explicable on this hypothesis. It is perhaps in place here to mention that apart from the inactivation curves of Baron et al. and my own, the published UV inactivation curves of vaccinia are represented as exponential (Collier et al., 1957; Hanafusa, 1960). Their results can be explained through faulty experimental techniques.

The usual explanations for multi-component inactivation curves are (a) a heterogeneity in the

sensitivity to radiation of the irradiated population, or (b) a heterogeneity in the substructure of each individual of the population.

The first has been shown not to explain the vaccinia inactivation curves; the second is untestable.

An attempt to explain the inhomogeneous UV inactivation curves of transforming deoxyribonucleic acid of bacteria (Lerman and Tolmach, 1959) has been made by Rupert and Goodgal (1960). They found that if the data were plotted as the $\log_{10}$ of the transforming ability against $\log_{10}$ of the UV dose, a straight line was obtained. Such an inactivation curve would indicate a power law inactivation rather than the target theory exponential inactivation. When the vaccinia data were plotted against the $\log_{10}$ of the UV dose, a straight line was not obtained.

Another recent attempt to explain such curves has been made by Pichl and Wecker (1960). Their theory is also applicable to inactivation curves of the multi-hit type. In sum, their hypothesis is that irradiation may cause not only direct lethal hits, but also some which are not directly concerned with survival, and only render the target <u>more</u> (convex curves) or <u>less</u> (concave curves) sensitive to the next hit. A mathematical model was derived which could be tested experimentally, and their experiments with phage T3 appeared to support the model.

However, it was not possible to differentiate between the direct and indirect modes of inactivation.

Preliminary experiments (Holmes, 1961; Abel, 1961a) have indicated that the cationic strengths of the diluent in which vaccinia virus was irradiated affected the shape of the UV inactivation curves. It might therefore be possible to adapt the Pichl-Wecker model to a study of the effects of the ionic strengths of the diluent on the inactivation of vaccinia.

The flattening-out of the inactivation curves of T-even phages (Luria, 1947) was found to be due to the phenomenon of multiplicity reactivation. A similar conclusion was reached for vaccinia virus.

The vaccinia strains tested all had about the same sensitivity to UV irradiation, but the sample tested was not representative of all members of the pox virus group. Nevertheless, there was no evidence of the two-fold difference in sensitivity found between T2 and T4, which was shown (Streisinger, 1956) to be linked to a single genetic locus u.

(c) Host Killing.

Qualitative experiments have shown that irradiated vaccinia virus which is unable to replicate is capable of killing chick embryo fibroblasts. The experiments also indicated that there were more cell-killing units than

pock infective forming units originally. Whether each physical particle is capable of killing a host cell cannot be stated definitely until quantitative studies are performed. Luria and Delbrück (1942) found that one UV-inactivated T2 phage was sufficient to kill the bacterial cell.

In contrast to the situation with vaccinia and T2, Marcus and Puck (1958) found that UV-inactivated NDV was not lethal to the host cell, whereas active virus was.

Although qualitatively the "toxic" effects of irradiated vaccinia virus appeared a rapid process, in reality the lethal events must have a time sequence such that it is possible for various 'reactivation' phenomena to occur before complete destruction of the cell. That is, irradiated virus must inhibit self-synthesis of the cell, but within a restricted time period, not potential virus synthesis.

(d) Interference.

The demonstration by Luria and Delbrück (1942) that pre-infection with UV-irradiated T2 made it impossible for viable T1 particles to multiply within the bacterial cells, is similar to qualitative results found with vaccinia. Quantitative studies in fibroblasts could not be made with vaccinia because of the cell-killing effect of irradiated virus. However, inoculation of heavily

irradiated virus together with suitable dilutions of unirradiated virus onto the CAM resulted in more than a 2 log drop in the titre of the unirradiated virus. Matumotu and Shinkawa (1956) have reported interference of active vaccinia on the CAM by prior inoculation of irradiated vaccinia.

With influenza virus where heterologous interference is a well studied phenomenon, it should be mentioned that in cross reactivation studies (Gotlieb and Hirst, 1956), irradiated virus of the same type could be "rescued" at least 16 hours later by a second inoculum of live virus.

(f) Multiplicity Reactivation.

Multiplicity reactivation has been demonstrated for the T-even phages and for T5, which in most respects behaves as a T-even (Luria, 1947; Luria and Dulbecco, 1949), and Tessman and Ozaki (1957) have shown a low level of MR for T1. This low level is due to the superimposition of the role of the bacterial cell in which MR occurs. The poor or non-reactivability of the odd phages is a necessary consequence of their partial genetic homology with the host, for if the host genome is unable to reactivate such a phage because of the high UV-dose the latter has received, the probability of reactivation through another equally hit phage will only be slightly increased. The mechanics of multiplicity reactivation in

the T-even phages have been discussed by Epstein (1958) and Barricelli (1960). Such detailed formal analyses are not yet possible with the vaccinia system because of the inability to express multiplicity in absolute terms.

The role of topography in the demonstration of MR with vaccinia is of great interest. Although Harm (1956) makes the comment that the distance between phage particles in a filamentous cell would reduce the probability of recombination, Bresch (1959) is the first virologist to realise the importance of topography in viral replication. Trautner and Bresch (1960), quoted in Bresch (1959), found, contrary to expectation, that increasing input multiplicities in recombination experiments with T1 and P22 resulted in an increase in recombinants. Topography, however, is an unforeseen complication in both the Visconti-Delbrück (1953) and Edgar-Steinberg (1958) theories of T-even phage recombination, and no solution to the problem has yet been achieved by the bacteriophage virologists.

With vaccinia virus, however, the concept of topography in genetic interaction opens a new field. For example, in kinetic studies, one would expect that recombinants would be found earlier after an inoculum of clumped reactants than after a dispersed mixed inoculum. Also, a difference in kinetics should be observable

between experiments in cells of different sizes.

(g) Cross Reactivation.

Cross reactivation, unlike multiplicity reactivation, is not confined among the bacteriophages to the T-even group. Rescue of markers from irradiated parents by a related active parent has been demonstrated for λ by Jacob and Wollman (1955), and P22 (Garen and Zinder, 1955), although with less efficiency than with the T-even group (Luria, 1947; Doermann et al., 1955; Krieg, 1959).

The rescue of the $\underline{u}^+$ marker from irradiated RP $\underline{u}^+$ by either of the mutants u 1 or u 2 appears to have a similar inactivation curve to the rescue of the $\underline{r}^+$ by an r mutant bacteriophage. That is, with increasing UV radiation it would appear as if the size of the target, the $\underline{u}^+$ marker, decreases, until a limiting size is reached (Krieg, 1959).

It is to be expected that the rate of inactivation of the marker would be much less than the rate of inactivation of the infectivity of the whole organism, and such results were found with T4 and vaccinia.

The detailed analysis performed by Krieg (1959) on the mechanics of rescue of the $\underline{r}II^+$ marker cannot yet be performed with vaccinia. Until the adsorption of u 1 and u 2 to KB cells can be improved, or another differential assay system perfected, only the high UV dose region can

be examined. As the probability of rescue at this level is small, anyway, further investigation of this region would be of little value.

The cross reactivation studies made with influenza virus (Gotlieb and Hirst, 1956; Baron et al., 1956; Fraser, 1959) all used the virulence marker. This most certainly is not a point marker (Fenner and Cairns, 1959) which has been adequately characterized, and although it may be readily assayable, it definitely cannot be satisfactorily quantitated. Although the papers of the influenza type are of interest to demonstrate how widely spread the phenomenon is, they add little to the deeper understanding of the mechanics of the genetic interaction.

(h) <u>Induction of Mutations</u>.

The potential mutagenic activity of UV radiation has been studied by several bacterial virologists, but the design of their experiments has often made the interpretation of their results equivocal. With those phages where there is a possibility of a partial genetic homology with the host, the reports of the appearance of mutants after UV radiation (Weigle and Dulbecco, 1953; Tessman, 1956; Fraser, 1957) could be explained by some form of contribution by the host genome to repair the damaged phage genome. Indeed, Bertani (1960) accepts this hypothesis as an explanation for the induction of

mutations in phage P2 by UV. Then, reports of increased mutation frequencies with T-even phages (Latarjet, 1949; Krieg, 1959b) could all involve the operation of multiplicity reactivation between irradiation and the expression of the mutation. Latarjet found that host range mutations could be induced by UV when T2-infected bacteria were irradiated whilst the phage was in the vegetative phase of multiplication. Krieg found that T4 $\underline{r}_{II}$ mutants, irradiated extracellularly, showed an increased frequency of reverse mutation to r_{II}^{+} when the bacteria were multiply infected. Again, vaccinia virus tends to resemble the T-even phages in that multiplicity reactivation would appear to enhance the expression of the mutagenic action of UV radiation.

"In general, this study points to the many similarities in the process of infection by vaccinia and the virulent phages. Admittedly vaccinia infection does not prevent the nucleus from starting its own DNA synthesis at the normal time, but this immunity of the nucleus may merely reflect its relatively isolated state in an animal cell. In other respects vaccinia infection may prove, in its essentials, much like phage infection but greatly spread in time just as it is spread in space."

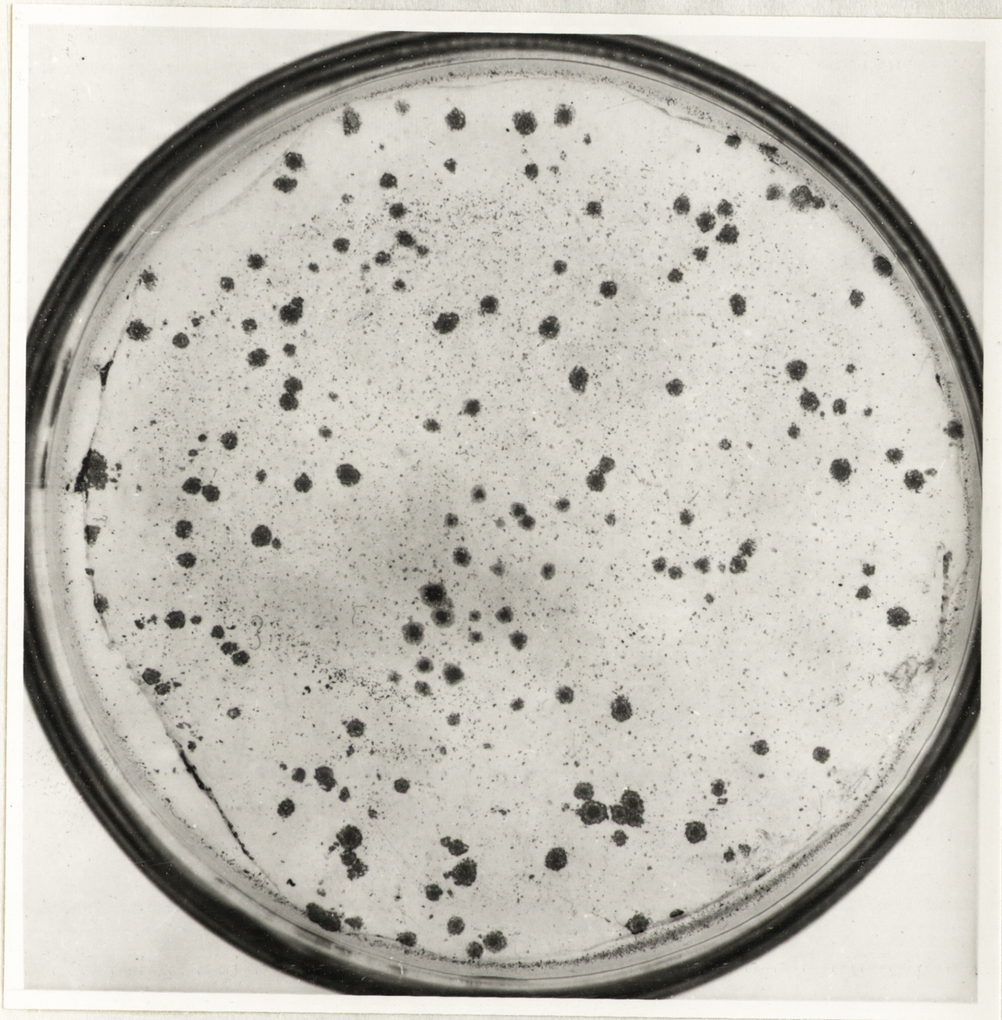
If one substitutes the word "radiobiology" for "process of infection" in the first sentence, and for "infection" in the final sentence, the above quotation of the final paragraph of a paper on the initiation of vaccinia infection (Cairns, 1960) is, in its essentials, both an apt summary of this thesis and a very good prediction of the results obtained. Although Cairns was more concerned with the time parameter in his paper, he does comment on the space, or topography, paramater. In this thesis, topography has been given major emphasis and shown to be a factor of significance in viral genetics.

APPENDIX

I. VIRUS AND INFECTIVE CENTRE ASSAYS.

For many years, one of the major problems in virology has been the titration of virus particles. There are two aspects to this problem - (1) the enumeration of the total number of virus particles in a preparation, and (2) the enumeration of the number of these particles which are infective in the assay system in use. The more sensitive the system of assay for infective particles, the lower will be the ratio between the two counts. When the virus under investigation has a characteristic shape and size, and when reasonably pure samples can be prepared, the direct count from electron micrographs is the most satisfactory method of obtaining the total number of virus particles in a preparation. Fortunately, as vaccinia virus has a very distinctive appearance under the electron microscope, and relatively pure preparations of virus can be formed from genetron extracts of infected chorioallantoic membranes, total counts can readily be obtained.

Virus Assays. The usual method for obtaining the infectivity titre of vaccinia virus is by titration on the chorioallantoic membrane (CAM) of the embryonated hen's egg. Following the development of tissue culture plaque techniques it was found (Noyes, 1953) that vaccinia virus could also be titrated on chick embryo



<u>Figure 1</u>. Haemadsorption assay with VMH virus.

Chick embryo fibroblasts were infected in suspension, and the monolayers stained 2 days later with vaccinia-sensitive fowl red cells.

fibroblast monolayers.

Inoculation of suitable dilutions of vaccinia virus on the CAM results in the formation of small pocks, each pock being the result of infection of the ectoderm by one virus particle. Suitable tissue culture assays on the other hand result in the formation of plaques within the matrix of a monolayer of cells. Each plaque, too, would appear to be the result of infection of the cells with a single virus particle. In this investigation chick embryo fibroblasts were used for the plaque assay, either as monolayers or as primary suspensions.

Use can also be made in CEF tissue culture of the production of a haemagglutinin by most vaccinial strains (Soloviev and Mastiukova, 1958). The virus was added to the primary cell suspension, but the medium was left on for two days. 0.2 ml. of a 10% suspension of vaccinia-susceptible fowl red blood cells was added to the plates and allowed to react for 2 hours at 37° C. The medium was then removed and the 'plaques' of red blood cells counted. Figure 1 shows a plate infected with VMH virus and stained with fowl red blood cells.

A statistical analysis of these four methods of titration was made using VMH virus. An 0.1 inoculum was used for all titrations. When more than 30 replicates were recorded for a particular titration method,

Table 1

SUMMARY OF VMH ASSAYS

Day		CAM	Method of Assay: Haemadsorption Plaques	Primary CEF	Monolayer of CEF
1.	$\bar{x}$[a]	-	11.2	14.7	3.1
	v		6.1	19.0	2.2
2.	$\bar{x}$[b]	229.0	22.0	-	-
	v	773.8	-	-	-
3.	$\bar{x}$	452.0	42.5	25.0	14.7
	v	1850	185	-	-
4.	$\bar{x}$	367.0	-	13.0	3.0
	v	831	-	-	-
5.	$\bar{x}$	350.0	26.0	-	4.4
	v	-	46.4	-	3
6.	$\bar{x}$	200.0	17.3	-	4.2
	v	-	12	-	4.6
7.	$\bar{x}$	182.0	9.6	15.4	1.9
	v	1433	7	126	1.7

(a) $\bar{x}$ = mean

(b) v = variance

Table 2

TRANSFORMATION OF TABLE 1 TO A LOGARITHMIC FORM

(This Table includes values missing from 1)

Day	Method of Assay			
	CAM	Haemadsorption Plaques	Primary CEF	Monolayer of CEF
1.	2.26	1.05	1.17	0.49
2.	2.36	1.34	1.12	0.54
3.	2.66	1.63	1.40	1.17
4.	2.56	1.20	1.11	0.48
5.	2.54	1.41	1.25	0.64
6.	2.30	1.24	1.11	0.62
7.	2.26	0.98	1.19	0.28

Table 3

ANALYSIS OF VARIANCE OF RESULTS IN TABLE 2

Source	df	S.S.	V.	F.	P.
Host	3	12.0282	4.0094	211.021	<0.001
Days	6	0.7406	0.1234	6.494	<0.001
Interaction	18	0.0190			

the variances are also given. A summary of the means and variances is given in Table 1. Because of the missing values in this Table, one cannot perform an analysis of variance on the results as given. Moreover, not only are there missing values, but the variances are also heteroschedastic. Therefore a schedasticity transformation must be performed before proceeding to an analysis of variance. This is done by converting the results to a logarithmic form as in Table 2, where also the missing values have been estimated according to the method described in Finney (1952). Table 3 gives the analysis of variance of the results.

It is evident that both the host and day effects are significant beyond the 0.1% level. It was to be expected that there would be a significant host effect, but not that there would be a day effect. An explanation can, however, be offered for this latter result. As the sets of readings move almost parallel over the days, the making up of the initial inoculum could be the cause of the variation between days. The significance is due largely, however, to the discrepantly high and low values of the monolayer readings on days 3 and 7 respectively - the rest differ by less than 0.1 log between days. On day 3, the monolayer readings are the mean of 3 readings only, and these particular plates

were in very poor condition for counting, there being many non-specific tears present.

Although the CAM counts are more efficient than the tissue culture counts, their consistently larger variance was considered unsatisfactory for accurately detecting small changes due to the effects of radiation. Haemadsorption counts, although having reasonably small variances, and giving an answer within 2 days as against 5 days with overlaid tissue cultures, could not be used for RP assay. Since RP was the strain used in all the studies of the genetic effects of radiation, primary CEF suspensions were employed for most of the assays, though their variance was somewhat larger than that of haemadsorption or monolayer assays. Although monolayer assays had low variances, they were too insensitive for efficient assay procedures.

Infective Centre Assays. The term infective centre was first used by Ellis and Delbrück (1939) in a paper on the growth of bacteriophage. They defined the terms infective centre and efficiency of plating as follows: "In the plaque counting method a single phage particle and an infected bacterium containing any number of phage particles will each give only one plaque. This method, therefore, does not give the number of phage particles but the number of loci within the solution at which one

or more phage particles exist. These loci will hereafter be called 'infective centers'.......We shall call the fraction of the infective centers which produce plaques the 'efficiency of plating'. ....this coefficient is essentially the fraction of infected bacteria in the suspension spread on the plate which goes through to lysis under our cultural conditions......". In bacteriophage work the plan of an infective centre experiment is to adsorb phage to host bacteria for a certain time, at the end of which the culture is treated with anti-phage antibody to neutralize any unadsorbed phage. The culture is washed, then diluted out to a level where a known volume of the dilution, when plated by the agar layer technique (Adams, 1959), will contain infective centres to produce a countable number of plaques. The efficiency of plating of phage infective centres is unity.

In animal virology, infective centres have until recently been used mainly to determine the fraction of a population of cells which have been infected. Their use for other than growth cycle studies has been very restricted; there are two reports on genetic aspects: a study of multiplicity reactivation with poliovirus (Drake, 1958), and a study of mixed infections with different strains of poliovirus or influenza virus

(Hirst, 1959). Three reports on the radiobiology of virus-cell complexes have been published: Franklin (1958) using vesicular stomatitis virus, Rubin and Temin (1958) using Rous sarcoma virus, and Powell (1959) using Herpes simplex virus.

Infective centres are most commonly prepared by adding virus to a monolayer of a particular cell line. After an adsorption period, varying from thirty minutes for the polio-monkey kidney system (Howes, 1959) to three hours for the herpes-rabbit kidney system (Kaplan, 1957), the monolayers are dispersed into single cells by trypsin or EDTA, or a mixture of the two. Many authors remark on the damage caused to cells by this dispersal procedure. Howes (1959) found that his "fertile cell count" was raised four times by using a mixture of trypsin and EDTA rather than either agent alone. The mixture dispersed the cells in a much shorter time, which may have resulted in less damage to the infected cells. Maitland and Postlethwaite (1959), working with vaccinia virus in HeLa cells, found that up to 50% of the cell-associated virus was released into the supernatant when the infected monolayers were treated with trypsin or EDTA. Whatever caused the release of this virus, titration of the "infected" cells would result in a very low infective centre count. Nevertheless, with some virus-cell systems,

this method of production of infective centres has given an efficiency of plating of 1. Stoker (1958) found that a herpes-infected HeLa monolayer, when treated with EDTA and plated in dilution on HeLa monolayers, yielded the expected number of infective centres. Franklin (1958), working with the VSV-chick embryo fibroblast system, was able to obtain a plating efficiency of 1 when chick fibroblast monolayers were used for assay, whereas Cooper (1955) could obtain only an efficiency of 0.2 to 0.4 with the same system.

If infective centres are formed when the cells are already in suspension, there is no trauma caused by dispersion after infection. Nevertheless, damage caused to the cells during the dispersion procedure before infection with virus is not eliminated, and it is probable that virus particles will adsorb on to a cell which will be unable to proceed with a complete cycle of virus growth. This virus-cell complex would not therefore resister as an infective centre when assayed.

Inhomogeneity of the cell strain used to produce the infective centre may also be a factor in the reported low efficiencies of plating. If only half the cells in the population are susceptible to virus growth, there can never be an efficiency of plating higher than 50%.

This might explain the frequent inefficiencies of plating when chick embryo fibroblasts are used as the infected cell.

The ability of an infected cell to plate as an infective centre may depend on the stage of growth of the virus within the cell when it is plated. Rubin (1960) was able to increase the efficiency of plating of Rous sarcoma virus-chick fibroblast infective centres by delaying the plating for 1 or 2 days, instead of plating immediately. Precautions were taken to ensure that the increased efficiency was not caused by later infection of previously uninfected cells.

Where penetration into the cell of adsorbed virus is slow, or where the washing procedure after adsorption does not effectively remove surface-attached virus, the virus-cell complexes may have to be treated with antibody to neutralize the major fraction of this virus. This is necessary, for, unless removed by washing or antibody treatment, this surface-attached virus will be able to initiate the formation of a pock or plaque when it comes in contact with the assay system, even though it does not originate from an infected cell.

Apart from a report by Hoggan and Roizman (1959) with Herpes-amnion system, the CAM appears to be an inefficient system of assay for infective centre. This is not wholly unexpected, for it is likely that the

environment of the CAM exhibits sub-optimal conditions for the production of potentially infective virus within an infected tissue-culture cell. This inefficiency of the CAM for the assay of infective centres is in direct contrast to its sensitivity for the assay of free virus. Stoker (1958) obtained a plating efficiency of 1 with Herpes-infected HeLa cells when assayed on HeLa monolayers, but only an efficiency of plating of 0.08 when assayed on the CAM, yet the CAM is more than ten times as sensitive for free virus as the 50% end point determined in HeLa . (A similar situation has been shown to apply to the vaccinia system.)

When assaying infective centres on monolayers, two characteristics of the virus strain in use must be considered - its size, and its heat stability. If an infected cell is swept up into the agar overlay as it is being applied, the size of the virus will determine its ability to diffuse down through the agar to the cell layer to initiate a plaque. For example, the limiting concentration of agar for the diffusion of poliovirus, 28 mμ in diameter, is 2.5%. However, even if the virus is small enough for diffusion, it must also be heat stable enough to diffuse through the agar without being inactivated by heat before it reaches the monolayer. Such considerations would limit the applications to be

made of the mash layer technique (Franklin, 1958) and the agar suspension of tissue culture cells (Cooper, 1955).

Keeping the relevant points of the previous discussion in view, a system of infective centre assays satisfactory for vaccinia virus was developed.

The system of infection of KB cells in suspension, developed by Easterbrook (1961) for growth curve studies, was adopted for infective centre assays, and when primary suspensions of CEF were used as the assay system, the efficiency of plating was consistently 1.

Before the cell infection method of Easterbrook was used in the infective centre assays, it had been found consistently that when the cells were treated with a vaccinial antibody after infection, the infective centre count dropped tenfold on a similar aliquot of cells untreated with antibody. This indicated that in systems without antibody treatment, some of the scored plaques could be formed from virus attached to the outside of the cells, and not to an infected cell. With the Easterbrook technique, however, it was found that antibody treatment was quite unnecessary.

The following experiment, which was designed to test the validity of the infective centre technique finally adopted, was performed in collaboration with Mr. K. B.

Easterbrook. Mr. Easterbrook prepared the KB cells and infected them with VMH virus at an input multiplicity of 4.6 pfu per cell. At 0, 4 and 6 hours after adsorption, an 0.1 ml. sample containing approximately 9 x 10^3 cells was added to 0.9 ml. of each of the following diluents:

(a) KB growth medium + 10% anti-vaccinial antibody = AG
(b) KB growth medium without antibody OG
(c) 0.5% calf serum in Hanks' BSS + 10% anti-vaccinial antibody AI
(d) " " " in Hanks' BSS without antibody OI

The tubes were incubated at 37° C for 20 minutes, after which the cells were centrifuged down and the cell deposit taken up in 5 ml. of the appropriate diluent. (It was noted that during this procedure, some cells were lost, in particular, the 4AI sample, and because of the low cell concentration, it would perhaps have been better had uninfected carrier cells been added prior to centrifugation). Dilutions were made, and 0.1 ml. volumes plated on the CAM, monolayers and primary CEF. Mr. Easterbrook made fluorescent antibody count of the cells at 4, 6, 8 and 30 hours, the results being given in Table 4. The results of the infective centre assay are summarized in Table 5, while an analysis of variance of these results, with an adjustment made for the spurious 4-hour AI values (where

Table 4

INCREASE IN PROPORTION OF CELLS REGISTERED BY FLUORESCENT ANTIBODY WITH TIME

Time of Sample	Percentage of Cells Staining
4 hours	28%
6 "	43%
8 "	56%
30 "	87%

Table 5

SUMMARY OF RESULTS OF INFECTIVE CENTRE EXPERIMENT[a]

Assay	CAM				CEF Monolayer				Primary Suspension of CEF			
Diluent[b]	AG	OG	AI	OI	AG	OG	AI	OI	AG	OG	AI	OI
Time of Sample												
0 hours	26	58	20	51	43	44	56	75	140	226	116	182
4 hours	14	25	(10)*	43	62	55	(34)*	74	186	195	(108)*	209
6 hours	17	42	29	35	48	44	61	47	221	200	195	206

*4AI Sample - cells lost in centrifugation.

(a) KB cells were infected in suspension with VMH virus at an input multiplicity of 4.6 pock forming units per cell.

(b) AG KB growth medium + antiserum

OG KB " " without antiserum

AI 0.5% calf serum in Hanks' BSS + antiserum

OI 0.5% " " " " " without antiserum

Table 6

ANALYSIS OF VARIANCE OF RESULTS IN TABLE 5

Source	df	S.S.	V	F	P
Host	2	164933	8246	396.5	<0.01
Time	2	498	249	1.2	--
Diluent	3	3235	1078	5.2	--
H x T	4	3248	812	3.9	--
H x D	6	3203	534	2.6	--
T x D	6	2746	457	2.2	--
H x T x D	12	2509	208		

an unknown number of cells were lost in the centrifugation) is given in Table 6.

This shows that the only significant interaction is between the method of assay, and that the time of assay and the diluent give no significant interaction. As the 30 hour fluorescent antibody stain, and the infective centre assay on primary CEF^s give the expected result, and the use of antibody makes no difference to the final infective centre count, it was felt that this experiment justified completely the use of this infective centre method in the radiobiological study of vaccinia virus.

II. INACTIVATION EXPERIMENTS.

If the energy of the UV light causing the inactivation of the virus does obey the quantum equation, $E = h\nu$, it should be possible to show whether the inactivation is primarily the result of a photochemical reaction. Since no temperature term appears in the equation, UV inactivation of virus should be independent of the temperature at which the irradiation is performed. Lederle-7N (Fenner, 1958) virus was irradiated in solutions kept at 4° C, 20° C and 37° C during the entire irradiation period, and until inoculation onto plates. The three inactivation curves (Figure 2) showed no divergence.

Again when the wavelength is kept constant, a change

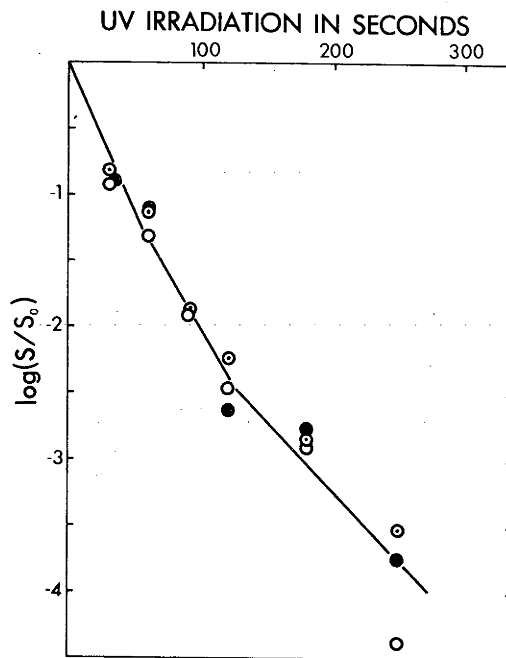
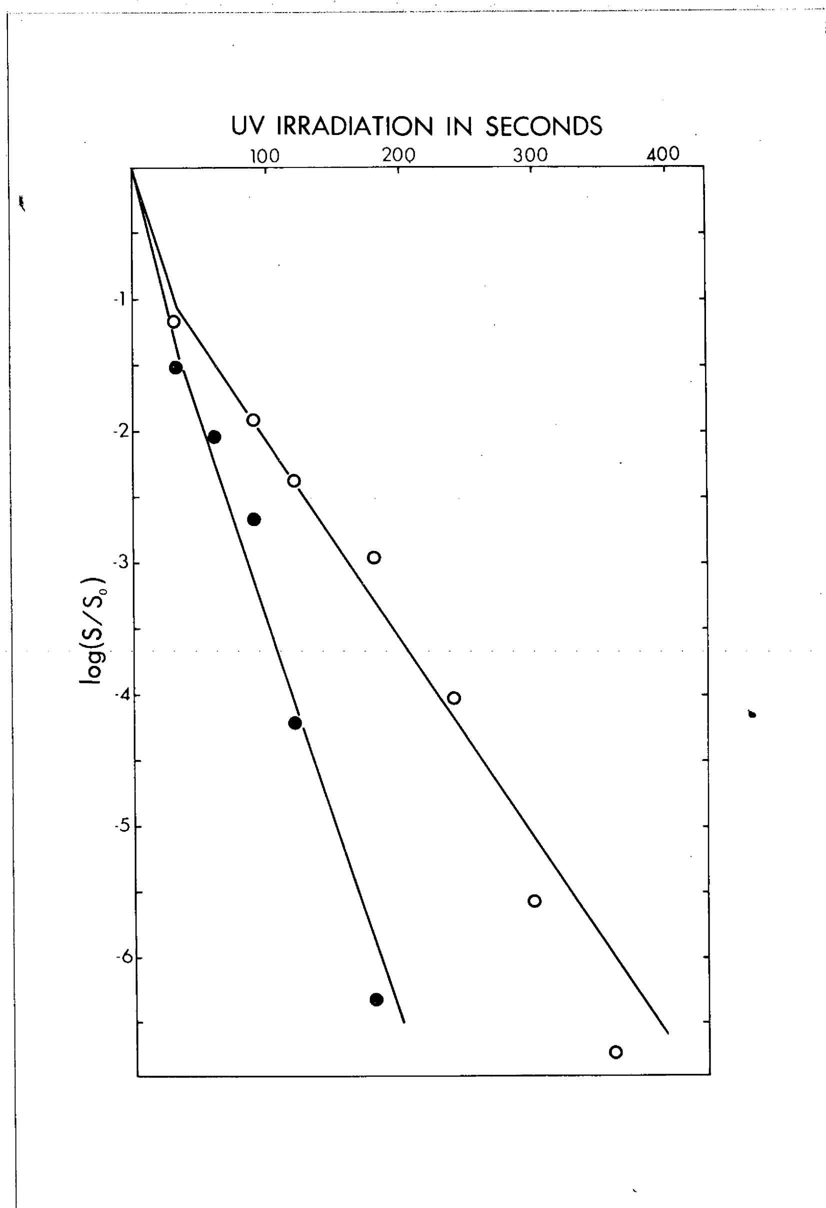


Figure 2. Effect of temperature on UV inactivation of 7N virus.

O Irradiation at 4° C.

● Irradiation at 20° C.

ʘ Irradiation at 37° C.



<u>Figure 3</u>. Dependence of inactivation rate on distance from the UV source. Samples of 7N virus were irradiated at 60 cm. (circles) or 43 cm. (dots), and assayed on CEF monolayers.

in the energy incident on the virus suspension would bear a direct relationship to the rate of inactivation. This is known as the Bunsen-Roscoe reciprocity law which states that the effect of exposure to radiation is a function of the total energy and is independent of intensity and time. The energy output is dependent on the square of the distance between the source of the light, and its object. Therefore, as 60 cm. was used as the standard distance between object and source, a distance of 43 cm. should result in twice the rate of the 60 cm. inactivation. That this was the case is shown in Figure 3, where the two curves are shown to be almost identical when the 43 cm. time scale is twice that of the 60 cm.

The inactivation of vaccinia virus by ultraviolet light is then primarily the result of a photochemical reaction.

While developing a standard irradiation technique, the effect of light and dark during the irradiation and assay procedures was investigated to see whether special precautions had to be taken to prevent photoreactivation. Aliquots of Lederle-7N vaccinia virus were irradiated both in daylight and almost total darkness. The assays on CEF monolayers were also done in either daylight or darkness. The results of a typical experiment are shown in Table 7. It can be seen that although the log

Table 7

EFFECT OF LIGHT AND DARK ON IRRADIATION AND ASSAY OF VACCINIA LEDERLE-7N

Irrad^n. time SECONDS	Dil^n.	Mean Plaque Count L[a]	Mean Plaque Count D[b]	$\text{Log}_{10}\frac{S}{S_o}$ L	$\text{Log}_{10}\frac{S}{S_o}$ D
0	-5	43	23	0.0000	0.0000
60	-4	23	9	-1.2718	-0.3075
120	-3	74	30	-1.7743	-1.8946
180	-3	9	6	-2.6793	-2.5837
240	-2	5	2	-3.9345	-4.0607
360	-1	1	-	-5.6335	

a: Virus was irradiated and assayed in daylight.

b: Virus was irradiated and assayed in almost total darkness.

survival values are almost identical for both assays, that is, no photoreactivation was observable, the absolute 'light' values are almost twice as high as the corresponding 'dark' values. The significance of this result is unknown.

(*Reprinted from Nature, Vol.* 186, *No.* 4729, *pp.* 992–993, *June* 18, 1960)

Reactivation of Poxviruses by a Non-Genetic Mechanism

IT has recently been shown that poxviruses inactivated by heat are reactivated in cells in which another poxvirus multiplies[1]. The virus yield from cells infected with inactivated virus of one strain and active virus of another strain contains infectious particles of both strains, and also, if the viruses are sufficiently closely related, recombinants[2]. Inactivation by heat, therefore, does not inactivate the deoxyribonucleic acid of the virus particles, since all the known genetic markers are preserved. Since proteins are in general less stable to heat than deoxyribonucleic acid, the role of the reactivating virus may be to provide some essential native protein which has been destroyed by heating. On this hypothesis, it should be possible to use, as the reactivating agent, virus of which the deoxyribonucleic acid has been specifically inactivated. In this case the genotype of the heat-inactivated virus only should survive.

Such inactivation might be achieved by treatment with the nitrogen mustard, di(2-chloroethyl)methylamine, which, although capable of combining with protein, reacts preferentially with nucleic acid. Loveless and Stock[3] have shown that nitrogen mustard inactivates phage probably by combination with its deoxyribonucleic acid without affecting the protein structures responsible for adsorption to host cells and combination with specific antibody, and they particularly comment on the low efficiency of multiplicity and cross-reactivation shown by phage treated with mustard.

Preliminary experiments showed that rabbitpox virus (RPu^+), treated with nitrogen mustard, would reactivate heated RPu^+, and also heated vaccinia 7N, and that the product had the pock character of the heated parent. In order to provide a sensitive test for partial survival of the genome of the mustard-treated parent, the mutants RPu1 and RPu8 were chosen as the heated parent. The u mutants of rabbitpox virus, which produce white non-ulcerated pocks on the chorioallantoic membrane, differ from the wild type (RPu^+), which produces hæmorrhagic ulcerated pocks, at single genetic sites[4,5]. In mixed infections of the chorioallantoic membrane with RPu1 or RPu8 and RPu^+, the wild type outgrows both mutants[5], so that if there is survival of that part

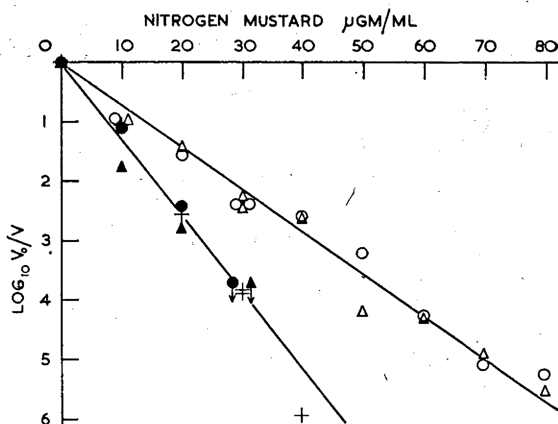


Fig. 1. Inactivation of RPu^+ by nitrogen mustard.
Assayed in the absence of heated virus: +, titre of U+ pocks.
Assayed in the presence of heated RPu1: ●, titre of U+ pocks; ○, titre of U pocks.
Assayed in the presence of heated RPu8: ▲, titre of U+ pocks; △, titre of U pocks

of the genome which carries the wild locus it should be readily detected.

RPu^+ was inactivated by exposure to various concentrations of nitrogen mustard in 0·005 *M* phosphate buffer, *p*H 7·2, for 20 min. at 37°. The excess mustard was then destroyed by a tenfold dilution of the suspension into 5 per cent sodium thiosulphate and further incubation for 2 hr. Assay of particles capable of reactivating heated virus was carried out by inoculating suitable dilutions of the material on the chorioallantoic membrane of 11-day old chick embryos together with 5×10^8 particles of *RPu*1 or *RPu*8 heated at 60° for 12 min.: the heated virus was completely non-infectious[1]. Residual active particles of RPu^+ were also titrated in the absence of heated virus.

The results are shown in Fig. 1. The infectivity of RPu^+ is seen to be destroyed by nitrogen mustard by a one-hit process, and the surviving particles can be assayed with equal efficiency in the presence or absence of heated virus. The ability of RPu^+ to reactivate heated RPu1 or RPu8 is destroyed less readily, though again this appears to be a one-hit process.

It seems, therefore, that in rabbitpox virus all the lethal damages caused by nitrogen mustard prevent expression of the genotype (as judged by one marker), but only about half of them fall in that part of the virus which is necessary for reactivation of heated

virus. Thus the evidence is compatible with our hypothesis, namely, that reactivating virus provides some essential non-genetic material which is lacking in heated virus.

We are indebted to Prof. Frank Fenner for the *u* mutants.

W. K. JOKLIK
PAMELA ABEL
I. H. HOLMES

Department of Microbiology,
John Curtin School of Medical Research,
Australian National University,
Canberra, Australia.

[1] Fenner, F., Holmes, I. H., Joklik, W. K., and Woodroofe, G. M., *Nature*, **183**, 1340 (1959). Hanafusa, H., Hanafusa, T., and Kamahora, J., *Biken's J.*, **2**, 85 (1959). Joklik, W. K., Woodroofe, G. M., Holmes, I. H., and Fenner, F., *Virology* (May 1960).

[2] Fenner, F., and Woodroofe, G. M., *Virology* (May 1960).

[3] Loveless, A., and Stock, J. C., *Proc. Roy. Soc. B*, **150**, 486 (1959). **151**, 148 (1959).

[4] Gemmell, A., and Cairns, H. J. F., *Virology*, **8**, 381 (1959).

[5] Gemmell, A., and Fenner, F., *Virology* (May 1960).

Printed in Great Britain by Fisher, Knight & Co., Ltd., St. Albans.

Acknowledgements

I wish to acknowledge my gratitude to Professor F. J. Fenner for suggesting this topic of research, and under whose supervision the investigation was made. I also wish to thank members of the Department of Microbiology, in particular, Professor S. Fazekas de St. Groth and Dr. H. J. F. Cairns, for their many stimulating and helpful discussions.

BIBLIOGRAPHY

Abel, P.M. (1961). Paper I.

Adams, M.H. (1959). Bacteriophages, Interscience, New York.

Baker, S.L., and Peacock, P.R. (1926). Brit. J. Exptl. Pathol. 7, 310-316.

Baron, S., Miller, A.H., Gochenour, A.M., and Hiatt, C.W. (1959). Fed. Proc. 18, 557.

Barricelli, N.A. (1956). Acta Biotheor. 11, 75-84.

Barricelli, N.A. (1960). Virology 11, 99-135.

Barry, R.D. (1960). Ph.D. Thesis, Australian National University, Canberra.

Bawden, F.C., and Kleczkowski, A. (1952). Nature 169, 90-91.

Bawden, F.C., and Kleczkowski, A. (1959). Nature 183, 503-504.

Bawden, F.C., and Pirie, N.W. (1937). Proc. Roy. Soc. B. 123, 274-320.

Bawden, F.C., and Pirie, N.W. (1938). Brit. J. Exptl. Pathol. 19, 251-263.

Benzer, S. (1952). J. Bacteriol. 63, 59-72.

Benzer, S. (1955). Proc. Nat. Acad. Sci. U.S. 41, 344-354.

Bertani, L.E. (1960). Virology 12, 553-569.

Bonhoeffer, F., and Schachman, H.K. (1960). Biochem. Biophys. Res. Comm. 2, 366-371.

Bowen, G.H. (1953). Cold Spring Harbor Symposia Quant. Biol. 18, 245-252.

Bresch, C. (1959). Ann. Rev. Microbiol. 13, 313-334.

Burnet, F.M. (1936). Spec. Rep. Ser. Med. Res. Coun. Lond. No. 220.

Cairns, J. (1960). Virology 11, 603-623.

Carnot, P., Camus, L., and Benard, H. (1926). Compt. rend. soc. biol. 95, 457-459.

Collier, L.H., McClean, D., and Vallet, L. (1955). J. Hyg. 53, 513-534.

Cooper, P.D. (1955). Virology 1, 397-401.

Delbrück, M., and Bailey, W.T. Jr. (1946). Cold Spring Harbor Symposia Quant. Biol. 11, 33-37.

Doermann, A.H., Chase, M., and Stahl, F.W. (1955). J. Cellular Comp. Physiol. 45, Suppl. 2, 51-74.

Drake, J.W. (1958). Virology 6, 244-264.

Dulbecco, R. (1950). J. Bacteriol. 59, 329-347.

Dulbecco, R. (1952). J. Bacteriol. 63, 199-207.

Dulbecco, R. (1952). Proc. Natl. Acad. Sci. U.S. 38, 747-752.

Dulbecco, R. (1955). In "Radiation Biology" (Ed. A. Hollaender), McGraw-Hill, New York, 2, 455-486.

Dulbecco, R., and Vogt, M. (1955). Ann. N.Y. Acad. Sci. 61, 790-800.

Dumbell, K.R., Downie, A.W., and Valentine, R.C. (1957). Virology 4, 467-482.

Easterbrook, K.B. (1961). In preparation. Virology.

Edgar, R.S., and Steinberg, C.M. (1958). Virology 6, 115-128.

Ellis, E.L., and Delbrück, M. (1939). J. Gen. Physiol. 22, 365-384.

Epstein, H.T. (1956). Bull. Math. Biophys. 18, 265-270.

Epstein, R.H. (1958). Virology 6, 382-404.

Fenner, F. (1958). Virology 5, 502-529.

Fenner, F., and Cairns, J. (1959). In "The Viruses" (Eds., F.M. Burnet and W.M. Stanley), Academic Press, New York, 3, 225-249.

Fenner, F., and Comben, B.M. (1958). Virology 5, 530-548.

Finney, D.J. (1952). Statistical Method in Biological Assay, Griffin, London.

Fisher, R., and McKinley, E.B. (1928). J. Infect. Dis. 40, 399-403.

Fogh, J. (1955). Proc. Soc. Exptl. Biol. Med. 89, 464-465.

Franklin, R.M. (1958). Virology 5, 408-424.

Fraser, D.K. (1957). Virology 3, 527-553.

Fraser, K.B. (1959). Virology 8, 544-546.

Friedberger, E. (1914). Berl. Klin. Woch. 51, 1402-1405.

Garen, A., and Zinder, N.D. (1955). Virology 1, 347-376.

Gates, F.L. (1934). J. Exptl. Med. 60, 179-188.

Gemmell, A., and Cairns, J. (1959). Virology 8, 381-383.

Gemmell, A., and Fenner, F. (1960). Virology 11, 219-235.

Gotlieb, T., and Hirst, G.K. (1956). Virology 2, 235-248.

Hanafusa, H. (1960). Biken's J. 3, 191-199.

Harm, W. (1956). Virology 2, 559-564.

Henle, W., and Henle, G. (1947). J. Exptl. Med. 85, 347-364.

Henle, W., and Liu, O.C. (1951). J. Exptl. Med. 94, 305-322.

Hershey, A.D. (1946). Genetics 31, 620-640.

Hirst, G.K. (1959). Symp. Soc. gen. Microbiol. 9, 82-101.

Hoggan, M.D., and Roizman, B. (1959). Virology 8, 508-524.

Hollaender, A., and Oliphant, J.W. (1944). J. Bacteriol. 48, 447-454.

Holmes, I.H. (1961). Personal Communication.

Howes, D.W. (1959). Virology 9, 96-109.

Huygens, C. (1690). Treatise on Light. Translated by S.P. Thompson, University of Chicago Press, Chicago, 1912.

Jacob, F. (1954). Compt. rend. 238, 732-734.

Jacob, F., and Wollman, E. (1955). Ann. inst. Pasteur 88, 724-749.

Kaplan, A.S. (1957). Virology 4, 435-457.

Kleczkowski, A. (1957). Advances in Virus Research 4, 191-220.

Kleczkowski, J., and Kleczkowski, A. (1953). J. Gen. Microbiol. 8, 135-144.

Krieg, D.R. (1959a). Virology 8, 80-98.

Krieg, D.R. (1959b). Virology 9, 215-227.

Latarjet, R. (1949). Compt. rend. 228, 1354-1357.

Lennox, E.S., Luria, S.E., and Benzer, S. (1954). Biochim. et Biophys. Acta 15, 471-474.

Lerman, L.S., and Tolmach, L.J. (1959). Biochim. et Biophys. Acta 33, 371-387.

Luria, S.E. (1947). Proc. Natl. Acad. Sci. U.S. 33, 253-264.

Luria, S.E., and Delbrück, M. (1942). Arch. Biochem. 1, 207-218.

Luria, S.E., and Dulbecco, R. (1949). Genetics 34, 93-125.

Luria, S.E., and Latarjet, R. (1947). J. Bacteriol. 53, 149-163.

Luria, S.E., Williams, R.C., and Backus, R.C. (1951). J. Bacteriol. 61, 179-188.

Maitland, H.B., and Postlethwaite, R. (1959). Symp. Soc. gen. Microbiol. 9, 185-199.

Marcus, P.I., and Puck, T.T. (1958). Virology 6, 405-423.

Matumoto, M., and Shinkawa, E. (1956). Compt. rend. soc. biol. 150, 1062-1066.

McKinley, E.B., Fisher, R., and Holden, M. (1926).

Proc. Soc. Exptl. Biol. Med. 23, 408-412.

Norman, A. (1960). Virology 10, 384-387.

Noyes, W.F. (1953). Proc. Soc. Exptl. Biol. Med. 83, 426-429.

Pichl, H., and Wecker, W. (1960). Z. Naturforsch. (B) 15B, 226-231.

Powell, W.F. (1959). Virology 9, 1-19.

Rivers, T.M., and Gates, F.L. (1928). J. Exptl. Med. 47, 45-49.

Rubin, H. (1960). Virology 10, 29-49.

Rubin, H., and Temin, H.M. (1959). Virology 7, 75-91.

Rupert, C.S., and Goodgal, S.H. (1960). Nature 185, 556-557.

Siegel, A., Wildman, S.G., and Ginoza, W. (1956). Nature 178, 1117-1118.

Soloviev, V.O., and Mastiukova, I.N. (1958). Problems of Virology 3, 370-374.

Stahl, F.W. (1959). In "The Viruses" (Eds, F.M. Burnet and W.M. Stanley), Academic Press, New York, 2, 353-385.

Stanley, W. (1936). Science 83, 626-627.

Stent, G.S. (1958). Advances in Virus Research 5, 95-149.

Stoker, M.G.P. (1958). Nature 182, 1525-1526.

Stoker, M.G.P., and Ross, R.W. (1958). J. Gen. Microbiol. 19, 250-266.

Streisinger, G. (1956). Virology 2, 1-12.

Streisinger, G., and Franklin, N.C. (1956). Cold Spring Harbor Symposia Quant. Biol. 21, 103-109.

Taylor, A.R., Sharp, D.G., Beard, D., Finkelstein, H., and Beard, J.H. (1941). J. Infect. Dis. 69, 224-231.

Tessman, E.S. (1956). Virology 2, 679-688.

Tessman, I., and Ozaki, T. (1957). Virology 4, 315-327.

Toomey, J.A. (1937). Am. J. Diseases Children 53, 1490-1491.

Trautner, T.A., Bresch, C. (1960). Z. Induktive Abstammungs. u. Vererbungslehre as quoted in Bresch (1959).

Twort, F.S. (1915). Lancet 189, 1241-1243.

Visconti, N., and Delbrück, M. (1953). Genetics 38 5-33.

Weigle, J.J. (1953). Proc. Natl. Acad. Sci. U.S. 39, 628-636.

Weigle, J.J., and Dulbecco, R. (1953). Experientia 9, 372-373.

Zelle, M.R., and Hollaender, A. (1954). J. Bacteriol. 68. 210-215.