

Observations on photodynamic inactivation of vaccinia virus and its effect on immunogenicity*

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(Received 10 March 1965)

INTRODUCTION

Raab (1900) first showed that living organisms could be sensitized to visible light in the presence of certain dyes. This dye-sensitized photodynamic action, or photoinactivation, was extensively discussed by Blum (1941). Perdrau & Todd (1933*a*) and Herzberg (1933) demonstrated dye-sensitized photoinactivation of several animal viruses. There are few studies on the antigenicity of photoinactivated viruses. Perdrau & Todd (1933*b*) and Dempsey & Mayer (1934) used photoinactivation for the experimental production of effective canine distemper vaccine. Burnet, Keogh & Lush (1937) showed that bacteriophages retained antigenicity after photodynamic inactivation with methylene blue. Galloway (1934) produced good immunity in rabbits with photoinactivated fixed rabies virus. After the start of the present work, Wallis, Sakurada & Melnick (1963) showed that both photoinactivated and formalin-inactivated influenza vaccines produced good immunity in mice. We have investigated some of the factors influencing the photoinactivation of the Lister Institute strain of vaccinia virus and the immunity of animals inoculated with photoinactivated material.

MATERIALS AND METHODS

Virus

For most of the experiments the Lister Institute strain of vaccinia virus was used. The virus was extracted from sheep dermal pulp in 0.004 M-McIlvaine buffer pH 7.2, and partially purified by treatment with 'Arceton 113' (trifluorotrichloroethane, I.C.I.), extraction with diethyl ether at 0° C., and differential centrifugation. The material usually had titres of 10⁷-10⁸ plaque-forming units per ml. and a total N₂ content of < 200 µg/ml. The virus was stored in small volumes at -70° C. and was rapidly thawed for use. Unused material was discarded. For some experiments the mouse neurotropic strain WR was used. This was propagated in chorioallantoic membranes (CAM) from dried mouse brain stocks, purified by a modification of the method of Joklik (1962*a*) and stored in small volumes at -70° C.

Virus assay

Virus was usually assayed by plaque counts in chick cell monolayer cultures in Leighton tubes without cover-slips or agar overlay. Some titrations were made

* Much of this work is incorporated in a Thesis to be presented to The University of London by G. S. T. for the degree of Ph.D.

by pock counts in CAM or by cytopathic end-point in rolled-tube cultures of monkey kidney cells (*Cercopithecus aethiops*). Titres are expressed as plaque-forming units (p.f.u.)/ml., or pock-forming units (po.f.u.)/ml., or 50% tissue culture infective doses (TCID₅₀)/ml. The chorioallantoic membrane and monkey kidney cells are sensitive indicators of vaccinia virus infectivity; both were used for the detection of very small amounts of virus.

Buffers

McIlvaine's buffer mixtures were used in most experiments. Virus dilutions for assay were made in diluted McIlvaine buffer at pH 7.2. Sørensen's phosphate buffer, Clark & Lubs borate + KCl + NaOH buffer and 0.01 M-tris buffer (1,3-propanediol, 2-amino-2-hydroxymethyl) were used in some experiments. All pH values were determined with a Model 23A pH meter (Electronic Industries Ltd.).

Methylene blue

Tetramethylthionine chloride (Methylene blue, May and Baker Ltd., 96% pure) was kept at room temperature as a 3×10^{-3} M aqueous stock solution. It was diluted for use to a concentration of 3×10^{-5} M, unless otherwise stated.

Illumination

The illuminant for photoinactivation was a Philips Photolita bulb connected to the mains through a variable resistance. Light was measured at the object level with a model S85 Weston photometer graduated in foot-candles (ft.-c.). All necessary manipulations were done under a safelight consisting of a domestic 40 W. bulb immersed in methylene blue solution of sufficient strength to give no meter reading at zero distance.

Photoinactivation

Reaction mixtures of methylene blue and suitably diluted virus in buffer were exposed in 9 cm. diameter glass Petri dishes with lids. The dishes stood upon a reflecting surface of aluminium foil. Ten ml. volumes gave a fluid depth of 1.5–1.7 mm. Occasionally larger volumes were used, giving depths up to 3 mm. After exposure for the required time, 0.5 ml. samples were removed and titrated. The removal of up to 3 ml. decreased fluid depth by < 0.5 mm. and increased light transmission by < 2%. In most experiments stock virus was diluted 100-fold giving an initial virus titre of 10^5 – 10^6 p.f.u./ml. and a total N₂ content of less than 2 µg./ml. Mixtures of diluted virus + methylene blue were limpid. In all experiments controls of unilluminated reaction mixtures (dark controls) and illuminated samples with dye omitted (light controls) were included.

Inhibition of photoinactivation

Inhibition was tested by including in the reaction mixtures suitable amounts of several substances.

Deoxyribose nucleic acid (DNA) and *ribose nucleic acid (RNA)* were commercial samples (British Drug Houses laboratory reagents) derived respectively from calf thymus and yeast.

Bases. Adenosine, thymidine, cytosine and guanylic acid (B.D.H.).

Proteins and derivatives tested were bovine crystalline albumin (BCA) Fraction V (Armour); a highly purified sample of human albumin kindly supplied by Mr L. Vallet of the Blood Products Laboratory at the Lister Institute; histidine monohydrochloride (L. Light and Co.); and calf serum (Oxoid Ltd.).

Enzymes

Peroxidase consisting of crude horse-radish extract, and a highly purified sample containing 102 units/mg., kindly given by Seravac Laboratories Ltd., were incorporated into the reaction mixtures in suitable amounts.

Catalase. Two samples were used, one a crude beef liver extract (L. Light and Co.); the other a more highly purified preparation containing 3000 units/mg. (Nutritional Biochemicals Corporation).

Ion exchange resin

Dowex A.G. 50 W.— $\times 8$ 200–400-mesh H form analytical grade (Bio Rad Laboratories) was converted to the Na form and equilibrated to the required pH by repeatedly washing with the appropriate buffer. Ten ml. volumes of virus + dye were shaken with 1.0 ml. volumes of packed resin in 50 ml. centrifuge tubes. This treatment rapidly removed visible dye without decreasing the virus titre of the sample.

Virus nucleic acid

Photoinactivated and untreated samples of vaccinia virus were concentrated by ultracentrifugation. The DNA was extracted by the method of Joklik (1962*b*), and estimated in a Unicam SP700 recording spectrophotometer, against a solution of thymus DNA 10 $\mu\text{g.}/\text{ml.}$ as standard.

Reactivation experiments

The methods described by Joklik, Woodroffe, Holmes & Fenner (1960) were closely followed. The WR strain of vaccinia virus has a large distinctive pock and is mouse neurovirulent. The Lister Institute strain of vaccinia virus produces compact well demarcated pocks and is *not* virulent for mice. These characters were used as easily distinguishable markers in reactivation experiments. WR had an initial titre of 5.6×10^8 po.f.u./ml., and 5×10^7 LD₅₀/ml. for mice by the intracerebral route. The Lister Institute strain had an initial titre of 10^9 po.f.u./ml. Samples of the viruses were thawed from -70°C. , given brief ultrasonic treatment to disperse aggregates, then either heated in sealed ampoules at 60°C. for 12 min., or photoinactivated, either minimally (7 min. at 50 ft.-c.) or for 20 min. at 50 ft.-c. After inactivation, excess dye was removed with ion exchange resin. Some samples were inactivated by brief exposure to ultra-violet light. The inactivated material was

used immediately. Its inactivity was tested in mice, CAM, or tissue culture. Reactivation experiments were done with combinations of undiluted variously inactivated material and suitable doses of live Lister virus. They were controlled with a mixture of undiluted heated WR (reactivable particles) and live Lister virus as reactivating agent. For the CAM, 30–50 po.f.u./dose of live Lister virus were used; for mice, the reactivating agent was increased to 10^4 po.f.u./dose. Reactivation in the CAM with the control material was readily observable. The identity of any doubtful pocks was checked by subinoculation to CAM or mice. Reactivation of control material in the mouse brain was readily detected by subinoculation to CAM and mice. Direct detection was not possible, probably because of interference by live Lister virus with the multiplication of WR in the mouse brain (Joklik *et al.* 1960). Brains were harvested for subculture on the fifth day after inoculation, a period adequate for the reproduction of infectious WR inoculated alone. In all experiments controls of the initial live virus of both strains were titrated. Ten 20 g. mice were used per group, with an intracerebral inoculum of 0.02 ml. In the CAM 6–8 embryos were used per group and the dose was 0.1 ml.

Preparation of experimental vaccines

Vaccinia virus was extracted from the dermal pulp of sheep, and bacterial contamination eliminated by treatment with phenol (0.6%, w/v). The virus was partially purified by treatment with Arceton 113 followed by differential centrifugation.

Vaccines A–C. Ten ml. volumes of vaccinia virus suspension with a titre of 10^7 TCD₅₀/ml. were mixed with methylene blue in a concentration of 3×10^{-5} M. They were then exposed in 6 in. Petri dishes to a light intensity of 50 ft.-c. for 10, 20 and 30 min. respectively. The methylene blue was not removed. The vaccines, labelled A, B and C, were stored at 4° C in bottles completely covered with aluminium foil to exclude light. Each vaccine was tested for residual infectivity by inoculating ten rolled-tube cultures of monkey kidney cells. All tubes were examined for cytopathic change after 1 week. With vaccine A 2/9 tubes showed cytopathic change. The culture fluid from these tubes was subinoculated, when 20/20 tubes were clearly positive. Though no cytopathic change was seen in the tubes inoculated with vaccines B and C, 20% of the pooled fluids of each group was subinoculated, with negative results. Unlike vaccines B and C, vaccine A, therefore, was not completely inactivated.

Vaccines D–F. These vaccines with initial titre of 5×10^8 po.f.u./ml. were inactivated the same way as vaccines A–C, except that exposures were for 20, 40 and 60 min. After illumination, the suspensions were treated with ion exchange resin and then centrifuged in an International Model PR-2 at 2000 rev./min. for 10 min. The dye-free supernatants were pipetted into separate bottles and stored at 4° C. Vaccine D was inoculated into 20 rolled-tube cultures of monkey kidney cells which were incubated for a week at 37° C., when 20% of the pooled culture fluid was subinoculated into fresh cultures and again incubated for a week; no cytopathic change was seen. Since no residual infectivity was demonstrable in vaccine D it could be safely assumed that vaccines E and F were similarly free of infectivity.

Immunization of rabbits

Vaccines A-C were each tested in three rabbits. The animals were bled and given an intramuscular injection of 1.0 ml. of vaccine. Fourteen days later they were bled again and given a second intramuscular injection of 1.0 ml.; 11 days later they were bled for the third time.

Vaccines D-F were each tested in five rabbits. The animals were bled and given an intramuscular injection of 1.0 ml. of vaccine. Nineteen days later they were bled again and given a second intramuscular injection of 1.0 ml. Thirteen days after the second dose they were bled for the third time. Serum samples separated from all the bleedings were inactivated at 56° C. for 30 min. and stored at 4° C. until neutralizing antibody was assayed.

Antibody assay

Neutralizing antibody was assayed by inhibition of plaque formation in monolayers of chick embryo cells. Dilutions of serum + standard challenge virus calculated to give 80-100 plaques per tube were incubated at 37° C. for 2 hr. in 10% skimmed milk in dilute McIlvaine buffer pH 7.2 (Boulter, 1957). The mixtures were chilled at the end of the incubation period and inoculated each into four cultures of chick embryo cells as for infectivity assays. A standard preparation of antivaccinial γ globulin made from pooled sera of hyperimmunized sheep was included in each group of assays. The standard was assigned an arbitrary potency of 100 units/ml. Dose-response curves of unknowns and standard were constructed in the usual way and the neutralizing potencies of immune rabbit sera were calculated in units.

Dermal challenge of immunized rabbits

Six weeks after the second dose of vaccine all the animals immunized with vaccines D-F were challenged by the application to the scarified skin of dilutions of a known potent smallpox vaccine. The animals were inspected daily and the extent of the lesions noted. The state of the lesions on the sixth day was taken as indicating the titre attained, as in potency assays of smallpox vaccine by rabbit dermal scarification. Observations, however, were continued until the eighth day, when any modification of lesions present (e.g. the presence of an eschar) were unequivocal.

RESULTS

Photoinactivation

A typical inactivation curve is shown in Fig. 1 where time was varied and intensity of illumination kept constant. When intensity was varied with time constant, similar curves were obtained. Data from such experiments plotted as logarithms of surviving fractions gave points falling closely about the same straight line (Fig. 2). The results indicate that: (1) the survival curve is exponential; (2) the rate of inactivation is determined by the intensity of illumination, and dose (intensity \times time) controls the degree of inactivation; (3) the reaction is probably of the 'single hit' type described by Lea (1955); and (4) at 50 ft.-c. very little virus

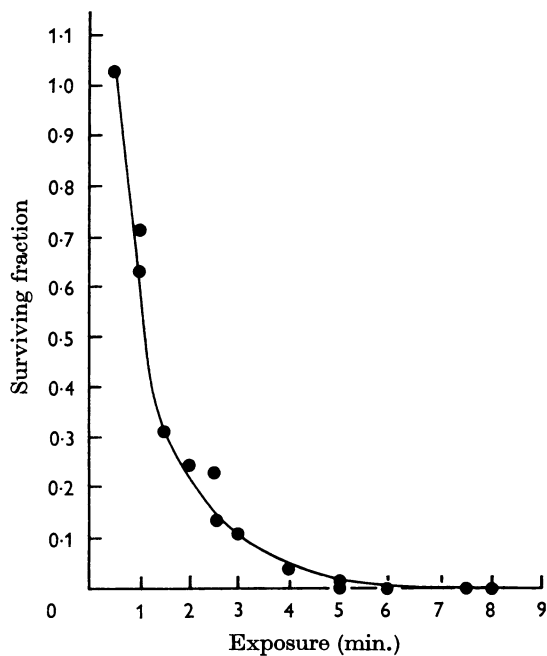


Fig. 1. Photoinactivation of vaccinia virus. Light intensity 50 ft.-c. 5.5×10^5 p.f.u./ml. virus in pH 7.0 buffer containing 3×10^{-5} M-methylene blue.

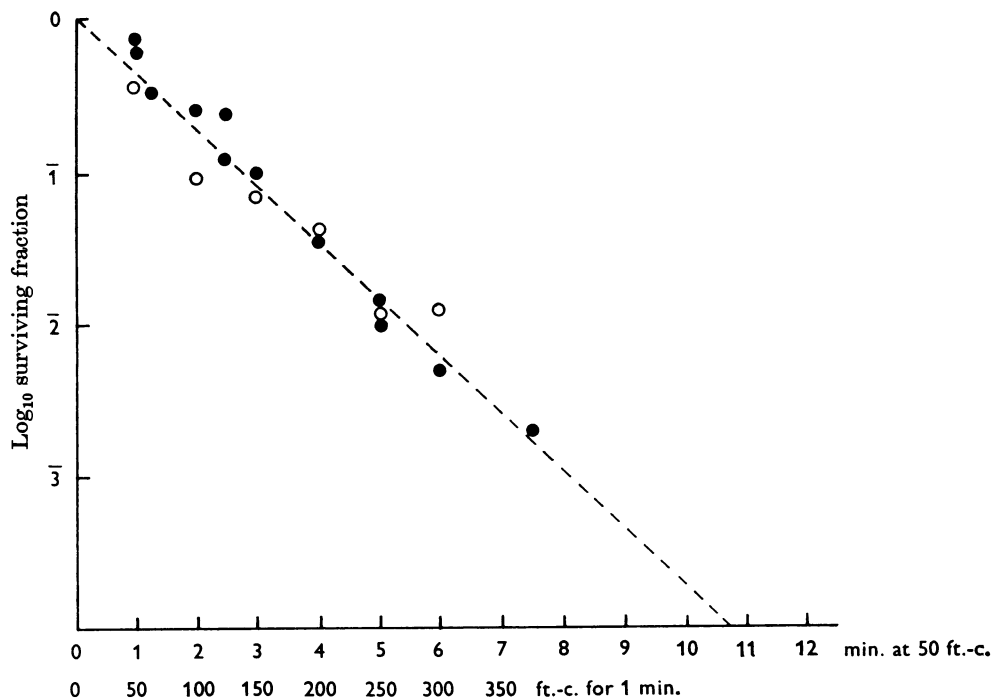


Fig. 2. Photoinactivation of vaccinia virus. Composite mean destruction curve. ● = 50 ft.-c. for various times. ○ = 1 min. at various intensities. 5.0×10^5 p.f.u./ml. virus in pH 7.0 buffer containing 3×10^{-5} M-methylene blue.

survived 10 min. exposure and none was detectable after 15 min. 'Blind' passage of undiluted photoinactivated material indicated that no multiplicity reactivation occurred. Nor did subsequent removal of methylene blue by dialysis, centrifugation or ion exchange resin reactivate already inactivated material.

Influence of methylene blue concentration

Inactivation was complete at approximately 10^{-5} M-methylene blue (Fig. 3). The standard concentration in most experiments was 3×10^{-5} M. Very high concentrations are said to mask photoinactivation in the deeper layers of a reaction mixture.

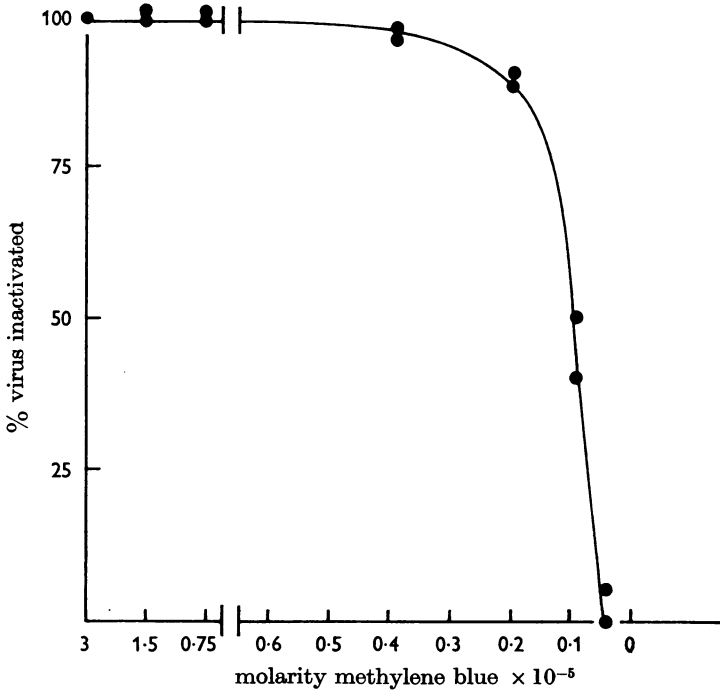


Fig. 3. Influence of methylene blue concentration on photoinactivation of vaccinia virus. 6×10^8 p.f.u./ml. virus in pH 7.0 buffer + various concentrations of methylene blue. Samples at each concentration exposed 10 min. at 50 ft.-c.

Influence of temperature

Most investigators have found photoinactivation to be virtually independent of temperature. Since low temperature might decrease reactivity we illuminated reaction mixtures at temperatures down to -20° C. (Table 1). Even at these temperatures reaction rates were not significantly affected. The presence of glycerol, used as an antifreeze agent, did however reduce the rates by approximately tenfold over the whole temperature range investigated, a phenomenon which is being examined further.

Influence of hydrogen-ion concentration

The photoinactivation of bacteriophages and some animal viruses depends on hydrogen-ion concentration (Welsh & Adams, 1954; Yamamoto, 1958; Wallis &

Melnick, 1963). Vaccinia is no exception; the rate increases directly with pH (Fig. 4). The pH affects the adsorption of dye to virus. Reaction mixtures were made in McIlvaine buffer at different pH values well within the stability limits of the virus (pH 5.0-9.0). After standing them at room temperature for 20 min. the mixtures were either centrifuged at 25,000 g for 30 min. and washed free of dye,

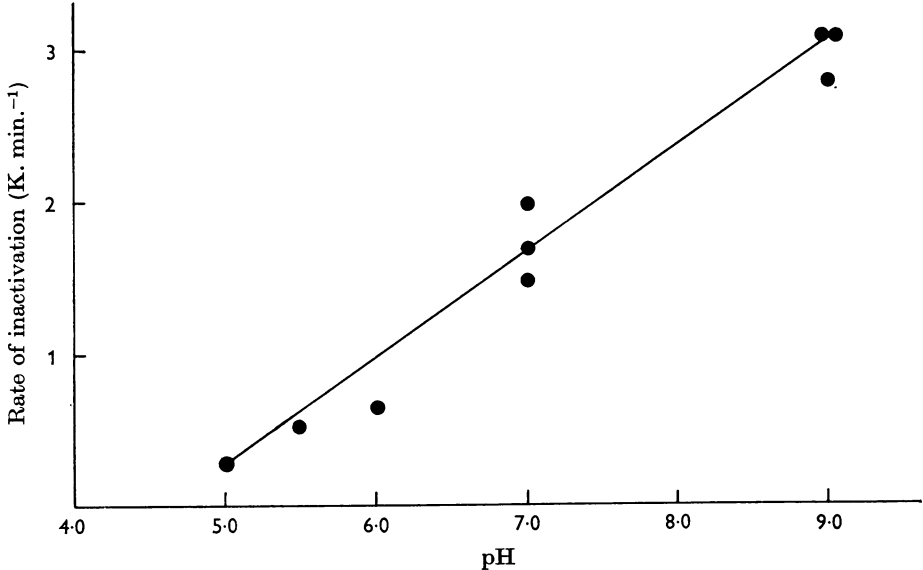


Fig. 4. Influence of pH on rate of photoinactivation of vaccinia virus. 10^5 p.f.u./ml. virus + 3×10^{-5} M-methylene blue, illuminated 50 ft.-c. for various times at different H ion concentrations.

Table 1. *The influence of temperature on photoinactivation of vaccinia virus in pH 7 buffer by 50 ft.-c.*

Temp. of Photoinactivation (°C.)	Velocity constant (K min. ⁻¹)	
	Buffer alone	Buffer + 40 % glycerol
-20	—	0.13
-10	—	0.11
0	1.46	0.11
20	1.42	0.12
37	1.55	—

or treated with ion-exchange resin equilibrated at the required pH values, all these manipulations being done in the safelight. On subsequent exposure to 50 ft.-c. for 30 min. photosensitivity was found to increase directly with pH (Fig. 5).

Methylene blue + virus mixtures were made at pH 9.0 and allowed to stand at room temperature. Free dye was removed by either ion-exchange resin or high-speed centrifugation or both and the virus resuspended in buffer at pH 5.0 and 7.0. During exposure to 50 ft.-c. the photoinactivation rates were slower than those of samples maintained at pH 9.0, but photosensitivity was not completely

abolished. Similar results were obtained by varying the test as follows. Methylene blue + virus mixtures were made at pH 9.0; free dye was removed with ion exchanger and virus sedimented by high-speed centrifugation. The sedimented virus together with suitable controls was resuspended in buffers at pH 5.0 and 6.0, shaken and treated again with ion-exchange resin. High-speed centrifugation was repeated, all the samples were resuspended at pH 9.0 and exposed to 50 ft.-c.

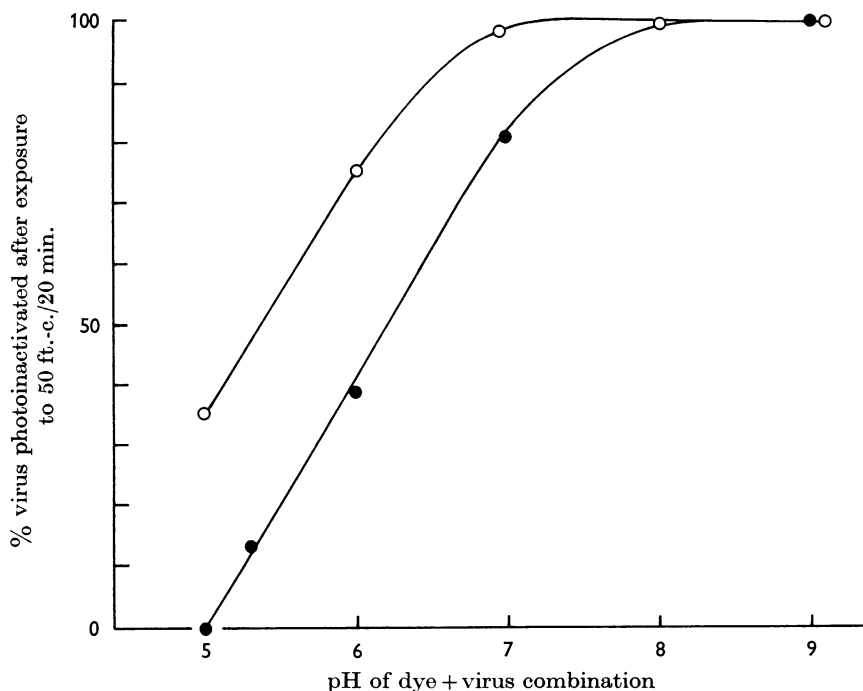


Fig. 5. Influence of pH on dye + virus combination. 3×10^{-5} M-methylene blue + 10^8 p.f.u./ml. virus allowed to combine at room temperature in dark at the pH values indicated. ○ = free dye removed with cationic exchange resin. ● = free dye removed by centrifugation. Colourless samples then exposed at 50 ft.-c. for 20 min.

The results indicate that the slow inactivation rate at low pH values is due to minimal combination of dye with virus; the attachment of dye to virus at low pH values is reversible; reversibility diminishes with increasing pH. No differences in inactivation rates were observed when reaction mixtures were prepared at the same pH in the three different buffers described under Methods.

Preincubation of methylene blue + virus mixtures

Mixtures of methylene blue + virus were made in pH 9.0 tris buffer and incubated at 0° C. or 37° C. for 1 hr. before removal of excess dye and exposure to 50 ft.-c. No significant differences in inactivation rates were observed. Since the temperature coefficient of methylene blue + vaccinia virus combination appeared to be negligibly small, mixtures were held at 0° C. for 10, 30, 60 min. and 24 hr. before removal of excess dye and exposure to 50 ft.-c. Again there were no differences in inactivation rates. Inactivation was always fastest in the presence of excess dye. At pH 9.0 removal of excess dye by ion exchange resin decreased the rate tenfold.

Influence of added nucleic acid and bases

Photodynamic action upon DNA and its guanine derivatives was reported by Simon & van Vunakis (1962, 1964). Since viral DNA is thought to be involved in photoinactivation, indirect evidence of DNA participation was sought by the addition of DNA and RNA solutions to vaccinia+methylene blue reaction mixtures. Both DNA and RNA inhibited photoinactivation, DNA more strongly than RNA (Fig. 6). Depolymerized samples of nucleic acid did not inhibit, nor did

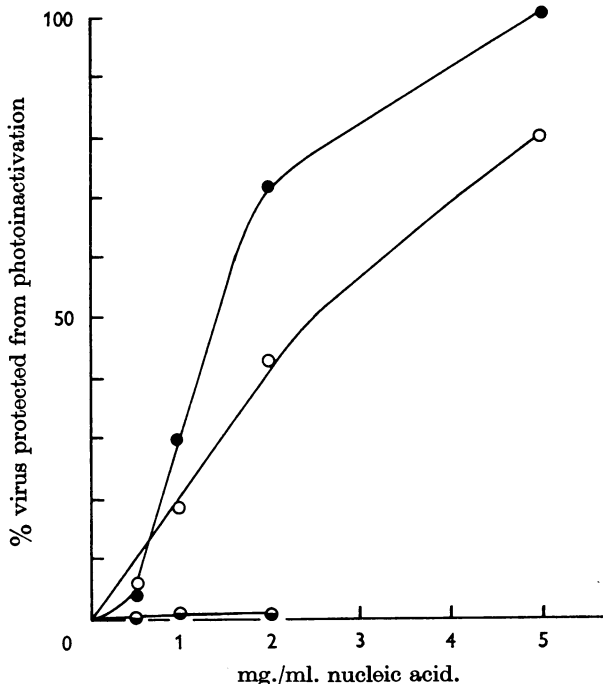


Fig. 6. Inhibition of photoinactivation by nucleic acids. ● = DNA; ○ = RNA; ◐ = depolymerized RNA.

the same concentrations of the individual bases or pentoses. Methylene blue has a peak absorption wavelength at approximately 700 m μ . Addition of nucleic acid to methylene blue reduced the total absorption and shifted the absorption maximum towards the red. The optical changes, however, occurred at DNA concentrations much lower than those with any measurable effect on photoinactivation. Methylene blue remained effective, and light at the altered wavelengths was still lethal in our reaction mixtures in the absence of added DNA. Experiments similar to those described for determining the effect of pH indicated that DNA too exerted its inhibitory effect by preventing dye-virus combination.

Influence of added protein

Since extraneous proteins often contaminate virus suspensions we incorporated into reaction mixtures bovine crystalline albumin, human serum albumin and whole calf serum. No inhibition of photoinactivation was observed when protein

was added in the same concentration as DNA; amounts in excess of 3% were required before the reaction rate was halved. Histidine (5 mg./ml.) did not prevent photoinactivation. The inhibitory effects of nucleic acid and large amounts of protein were not dependent on pH.

Influence of peroxidases

To test the possibility that photoinactivation might be mediated by transient peroxides formed during illumination of methylene blue + virus mixtures, we incorporated different samples of peroxidase and catalase into our reaction mixtures with suitable controls. Catalase up to concentrations of 6000 units/ml. and peroxidase up to 400 units/ml. were without effect.

Effect on viral nucleic acid

In attempts to determine whether photoinactivation is due to attack on viral DNA, nucleic acid was extracted from the virus and examined spectrophotometrically. In a typical experiment two 20 ml. portions of virus purified from rabbit dermal pulp were used. To one sample methylene blue was added. Both were exposed at 50 ft.-c. for 1 hr. The sample without dye had a titre of 3×10^8 p.f.u./ml.; there was no live virus in the photoinactivated sample. Nucleic acid was extracted from both samples by the method of Joklik (1962*b*). The photoinactivated sample yielded 100 μ g./ml. and the control 67 μ g./ml. The photoinactivated sample was diluted to the same DNA content as the control and both were scanned in the recording spectrophotometer. There were no spectral differences between the samples; nor were there any changes referable to a particular base when solutions of guanylic acid, adenosine, cytosine and thymine 10 μ g./ml. were scanned and recorded on the same plot.

Reactivation experiments

Cross-reactivation among suitably inactivated pox viruses is well established (Joklik *et al.* 1960; Fenner, 1962). The reactivation process requires that the

Table 2. *The influence of photoinactivation on reactivation of infectivity*

Potential reactivable material	Reactivating agent		
	Live Lister	Photo-inactivated Lister	Ultra-violet inactivated Lister
Heated WR	+	0	0*
Photoinactivated WR	0	nd	nd

+ = reactivation; 0 = no reactivation; nd = not done.

(Results summarized from four experiments in mice and CAM)

* In one experiment in CAM reactivation may have occurred, but could not be confirmed.

participating virus particles should have either protein or nucleic acid intact. We utilized this requirement in an attempt to determine the site of action of photoinactivation. The results are summarized in Table 2. In the control system of

heated WR and live Lister virus, reactivation readily occurred, both in mouse brain and CAM. Photoinactivated virus was not reactivable by live virus, nor was it capable of reactivating heated virus under similar conditions.

Immunogenicity of photoinactivated vaccinia virus

Vaccines A-C

Despite the presence of a small amount of residual infectivity in vaccine A the mean antibody concentrations in the sera of the animals immunized with it were only about twice those given by the non-infective vaccines B and C (Table 3).

Table 3. *Influence of duration of exposure on immunogenicity of photoinactivated vaccinia virus*

Vaccine	Exposure (min.)	No. of rabbits	Mean neutralizing antibody (units)		
			Initial	After 1 dose	After 2 doses
A	10	3*	0†	0.41	54.05
B	20	3	0	0.3	22.5
C	30	3	0	0.28	23.0

Titre of virus before inactivation = 10^7 TCD 50.

* One animal died before second bleed.

† 0 = no antibody detected in lowest dilution tested, i.e. < 0.25 unit.

Vaccines D-F

The antibody concentrations attained by the animals immunized with these vaccines (Table 4) indicate that apparent differences in immunogenicity between them are not due to exposure time. When the rabbits of the three groups were later

Table 4. *Influence of duration of exposure on immunogenicity of photoinactivated vaccinia virus*

Vaccine	Exposure (min.)	No. of rabbits	Mean neutralizing antibody (units)		
			Initial	After 1 dose	After 2 doses
D	20	5	0*	2.3	185.6
E	40	5	0	2.2	81.9
F	60	5	0	8.9	183.2

Titre of virus before inactivation = 5×10^8 p.f.u./ml.

* 0 = no antibody detected in lowest dilution tested, i.e. < 0.25 unit.

challenged by dermal scarification with potent vaccine a considerable degree of skin immunity was apparent (Tables 5-7), but it was not possible to distinguish between the three groups. Compared with the control animals, in all the immunized rabbits there was, by the 8th day, considerable acceleration in the evolution of the vaccinal lesions.

The large difference in the mean antibody response to the two groups of vaccines can be ascribed to their differences in virus content. Vaccines D-F had approximately 50 times the virus content of vaccines A-C.

Table 5. *Response to dermal challenge of rabbits immunized with vaccine D (20 min. illumination)*

Animal no.	Dilution of challenge vaccine $\times 10^{-3}$					
	1	3	9	27	81	243
Control	c	c	c	c	c	sc -
IR 10	sc	5	2	1	0	0
IR 11	7	6	1	0	0	0
IR 12	10	7	2	0	1	0
IR 13			Died before challenge			
IR 14	11	7	3	0	0	0

c, sc+, sc, sc- = respectively 100, 75, 50 and < 50% confluent lesion. Numerals = number of discrete pocks.

Table 6. *Response to dermal challenge of rabbits immunized with vaccine E (40 min. illumination)*

Animal no.	Dilution of challenge vaccine $\times 10^{-3}$					
	1	3	9	27	81	243
Control	c	c	c	c	sc -	1
IR 15	c	sc	1	0	0	0
IR 16	12	4	1	1	1	0
IR 17	4	3	1	0	0	0
IR 18	sc	4	2	0	1	0
IR 19	5	3	0	0	0	0

c, sc+, sc, sc- = respectively 100, 75, 50 and < 50% confluent lesion. Numerals = number of discrete pocks.

Table 7. *Response to dermal challenge of rabbits immunized with vaccine F (60 min. illumination)*

Animal no.	Dilution of challenge vaccine $\times 10^{-3}$					
	1	3	9	27	81	243
Control	c	c	c	c	sc	sc -
IR 20	sc +	3	2	3	0	0
IR 21	c	sc	1	0	1	0
IR 22			Died of intercurrent infection			
IR 23	6	5	1	0	0	0
IR 24	4	3	0	2	0	0

c, sc+, sc, sc- = respectively 100, 75, 50 and < 50% confluent lesion. Numerals = number of discrete pocks.

DISCUSSION

The dye-sensitized photoinactivation of vaccinia virus demonstrated here is similar to that of many other mammalian and bacterial viruses. It would be unwise however, to extend all the findings of one system to another. After Perdrau & Todd (1933*a*) first applied the method to animal viruses, Burnet (1933) recorded differing sensitivities to photoinactivation among serologically different members of the same group of bacteriophages. The external structure of the virus protein coat probably determines susceptibility to a particular dye by controlling either adsorption or permeability. The striking effects of pH on photosensitization may be concerned with virus protein in a manner similar to that reported by Stearn & Stearn

(1923) for bacteria. Our results follow from the basicity of methylene blue ($pK < 1.0$) and the increasing net negative charge of vaccinia virus protein with increasing pH (isoelectric point *ca.* 4.5) (Smadel & Hoagland, 1942). The binding of dye to virus around neutrality and below is probably ionic, since what little dye unites is readily detachable by cationic exchange resins. The irreversibility at high pH values may be due to firmer electrostatic bonding; or to modification of the virus protein facilitating dye penetration, a phenomenon utilized by Müller & Peters (1963) in the negative staining of subsurface structures in vaccinia virus for electron microscopy. Wallis & Melnick (1963) showed that vaccinia virus could be reversibly photosensitized. Subsequently they demonstrated irreversible photosensitization (Wallis & Melnick, 1964). Our experiments confirm both these findings and show that the photosensitization of vaccinia virus by methylene blue and its reversibility is determined largely by H ion concentration. We were unable to show an influence of time or temperature on the uptake or tenacity of binding of dye to virus before photoinactivation, unlike Helprin & Hiatt (1959) with coliphage and Wallis & Melnick (1964) with herpes virus. The removal of excess dye by ion exchange resin, centrifugation or dilution was always followed by a reduction in inactivation rate, suggesting the reversibility of combination of some dye and virus (Yamamoto, 1958).

The inhibition of photoinactivation by nucleic acid can be readily explained as the result of formation of complexes with dye (see Northland, De Bruyn & Smith, 1954; Peacocke & Skerette, 1956; Kay, Walwick & Gifford, 1964*a, b*). The protective effects of cellular material reported by Perdrau & Todd (1933*a*) may also have been due to nucleic acid. The inhibition of photoinactivation of poliovirus by cell fractions (Wallis & Melnick, 1963) also appears to be due to inhibition of the combination of dye with virus.

The amino acid histidine is principally attacked by photo-oxidation of protein (Weil, James & Butchert, 1953; Sajgó Mihály, 1963). At the concentration tested added histidine was without effect in our system. Whole protein was a less effective inhibitor than intact nucleic acid. The inhibition by nucleic acid, and possibly protein, could probably be effected non-specifically by many substances whose affinity for dye is greater than that of the specific receptor on the virus. Perdrau & Todd (1933*a*) discussed the oxygen dependence of photoinactivation and its possible mediation by a transient organic peroxide. However, Welsh & Adams (1954) could not correlate the oxidative effects of dye-sensitized photoinactivation with inactivation by agents like hydrogen peroxide. Our negative results with peroxidase and catalase also indicate that organic peroxide is not involved. It is generally assumed that photoinactivation is mediated solely through the modification of viral nucleic acid since the photosensitizing dyes are basic and have strong affinity for nuclear material. McLaren & Shugar (1964) emphasized the desirability of some direct evidence of the participation of nucleic acid in photoinactivation. Our attempts to provide such evidence were unsuccessful. Simon & van Vunakis (1962, 1964) and Sussenbach & Berends (1963) produced spectral changes in solutions of nucleic acid or bases by dye-sensitized photodynamic action, with light intensities that were much higher than ours. Under our

conditions, which inactivated vaccinia virus in a few minutes, 3 days were required to produce significant changes in a solution of guanylic acid, suggesting that the damage needed to inactivate intact virus and the quantities of material involved are probably far too small to be detected by conventional techniques.

Under controlled conditions of protein denaturation pox viruses are inactivated in a manner which leaves them reactivable. They may be reactivated by other active viruses of the same group or by virus inactivated by agents which selectively modify viral nucleic acid leaving protein intact (Joklik, 1964). Our results with photoinactivated material indicate strongly that vaccinia viral DNA is not the sole participant in photoinactivation but that protein too is involved. The results substantiate those quoted and demonstrated by Joklik (1964) although our experiments were not designed to support a hypothesis of inducer protein. McLaren & Shugar (1964) reviewed the ample evidence for photo-oxidation of enzymes and proteins sensitized by methylene blue. The cyclic amino acids are usually attacked and the effects are local in character leaving the general properties of the protein intact. It may well be for this reason that the antigenicity of vaccinia virus is spared.

SUMMARY

Photoinactivation of vaccinia virus sensitized by methylene blue had parameters similar to those observed with other viruses. Thus inactivation proceeded exponentially to completion, was irreversible, independent of temperature and the intensity of illumination. Inactivation was dependent on the dose of illumination and the concentrations of both methylene blue and hydrogen ions. The effects of pH appeared to be primarily concerned with the tenacity of dye-virus binding. Inactivation was inhibited by small amounts of nucleic acid but not by their bases or pentoses. Inactivation was only affected by the presence of extraneous protein in relatively high concentration: it was not affected by the enzymes catalase or peroxidase. Attempts to obtain direct chemical evidence of the participation of viral nucleic acid in photoinactivation were unsuccessful.

Recombination experiments strongly indicated the involvement of viral protein in photoinactivation. Immunogenicity was not impaired since good responses of neutralizing antibody were obtained in rabbits immunized with vaccines photo-inactivated over a wide range of exposure times.

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