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PHOTODYNAMIC INACTIVATION OF JAPANESE ENCEPHALITIS VIRUS

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SUMMARY Japanese encephalitis virus (JEV) is photodynamically inactivated when irradiated with visible light in the presence of proflavine (PF) in the extracellular state. The kinetics of the photoinactivation give a single hit curve both for JEV and for free JEV-RNA. It is impossible to extract the infectious RNA from the photoinactivated JEV by the cold phenol method. The photoinactivation reaction is oxygen dependent and shows no dependence on temperature (4°C–37°C). The absorption spectrum of PF has a peak at 445 mμ and a shift of this peak to 458 mμ occurs on the addition of yeast RNA to PF. The action spectrum for JEV photoinactivation by PF corresponds approximately to the absorption spectrum of a mixture of PF and yeast RNA but not to that of free PF, suggesting that the dye may be directly bound by the JEV-RNA moiety and the site of dye-sensitized photoinactivation may be viral RNA. The binding of PF by JEV seems to be rapid and reversible. The hemagglutinating (HA) activity of JEV is not affected by PF and visible light and photoinactivated JEV shows the same sedimentation pattern in a sucrose density gradient as normal JEV.

INTRODUCTION

Photodynamic inactivation of viruses, especially of poliovirus, by vital staining with the acridine series of dyes and visible light has been investigated by several groups of workers. As poliovirus and other enteroviruses can usually be photosensitized only when they are grown in the presence of dyes, it is considered that these dyes may be incorporated into the virions during their developing stages (CROWTHER and MELNICK, 1961., SCHAFFER, 1960, 1962., WILSON and COOPER, 1963, 1965). On the other hand, several viruses can be readily converted to the photosensitized state in the presence of dyes in the extracellular state. These viruses are members of the adeno-, papova-, pox-, reo-, and myxovirus groups (HIATT, 1960, WALLIS *et al.* 1963). It has been postulated by HIATT that the susceptibility of viruses to extracellular photoinactivation is due to a change in the permeability of the outer coat, or capsid, of the virus, to the penetration of dyes (HIATT, 1960). TOMITA and PRINCE reported that arboviruses of group A and B, to which JEV belongs, are highly susceptible to

extracellular photoinactivation by neutral red and visible light, and discussed the possible application of this method of inactivation to the production of killed virus vaccines (TOMITA and PRINCE, 1963). They also suggested in their paper, that from the observed first order kinetics of the photoinactivation, genetic rather than capsid damage of the virus occurred. However, there have been no reports giving evidence of the mechanism of the extracellular photoinactivation of viruses; that is, the problem is whether the dye can be bound directly by the viral RNA moiety and the light energy absorbed by the bound dye damage the viral RNA, or whether the dye is bound by the outer coat of the virus and the absorbed light energy is transmitted to the viral RNA moiety inactivating it. The present work was therefore carried out to obtain information on the mechanism of photoinactivation and on which component of the JEV particle is related to its photoinactivation.

MATERIALS AND METHODS

1. *Virus*

The virus used was the Nakayama strain of JEV which was grown in adult mouse brain. Infected mouse brains were homogenized with 9 volumes of 0.14 M NaCl in 0.02 M Tris-HCl (pH 7.6) containing 100 μg/ml of cystine (TCS), and centrifuged at $8,500 \times g$ for 15 minutes. From the resulting supernatant (S_1), partially purified JEV (U_2) was prepared by two cycles of differential centrifugation (90,000 $\times g$, for 90 min. for spinning down and at $2,000 \times g$, for 15 min. for clarification). In the successive experiments, U_2 or S_1 was employed. As previously described, the infectious RNA of JEV which was extractable from S_1 or U_2 by the cold phenol method is derived from mature, hemagglutinating JEV particles which sediment as a single peak in a sucrose gradient (IGARASHI *et al.* 1964).

2. *Extraction of RNA*

The cold phenol extraction procedure of GIERER and SCHRAMM (1956 a, b) was employed, followed by cold ethanol and 1 M NaCl precipitation to give further concentration and purification.

3. *Infectivity titration*

Infectivity of JEV and JEV-RNA was titrated by plaque forming activity (PFU) on a chick embryo cell monolayer, as described previously (IGARASHI *et al.* 1963).

4. *Irradiation by visible light*

Test tubes containing 2 ml of JEV or JEV-RNA suspension in TVS (0.02 M Tris-HCl pH 7.6, 1 mM EDTA, and 0.1 M NaCl) with or without PF were exposed to a 20 watt day-light type fluorescent lamp (FL 20 D Toshiba) at a distance of 2–10 cm at 4°C. When not being irradiated, samples were handled under a yellow safety lamp (FL 20 YF National).

5. *Absorption spectra*

Absorption spectra of free PF and a mixture of PF and yeast RNA were recorded in a Shimazu automatic recording spectrophotometer, type RS-27.

6. *Action spectrum*

As described previously (KONDO and KATO, 1966), the action spectrum for JEV photoinactivation by PF was obtained by using monochromatic light beams from a large-grating monochromator (BAUSCH and LOMB). The light source was a 500 watt high pressure mercury arc lamp (PHILIPS SP-500). Samples were placed in a quartz spectrophotometer cuvette (10 mm in optical distance) and stirred gently during the illumination. A filter of polyester film that transmitted poorly below 300 mμ was placed in the beam to eliminate light of lower wavelengths which might pass the monochromator and cause inactivation. The dose rates of the monochromator beams were determined with a d-c breaker amplifier (BECKMAN) and a thermopile calibrated against a National Bureau of Standards carbon-filament standard lamp. The action spectrum obtained, was compared with the absorption spectra described above.

7. *Sephadex gell filtration*

Two ml of a mixture of PF and JEV were placed on a Sephadex G-100 column (2 cm $\times$ 20 cm) and eluted with TCS at 5°C in the dark.

8. *Titration of hemagglutinating (HA) activity and the hemagglutination inhibition (HI) test*

HA activity of JEV and the HI test of the antiserum

against JEV were estimated by the method of CLARKE and CASALS (1958).

9. *Sucrose density gradient centrifugation*

A sucrose density gradient of 5–23% in TCS and one hour's centrifugation at 30,000 rpm in the SW-39 rotor of a Spinco model L were used.

10. *Chemicals*

Proflavine was a product of Tokyo Kasei Organic Chemicals and yeast RNA was commercial grade material from Sigma Chemical Co. U. S. A. This RNA was precipitated with 2 volumes of cold ethanol and the precipitate was washed three times with 67% ethanol.

11. *Antiserum*

Anti-JEV rabbit hyperimmune serum was kindly supplied by Dr. C. H. LEE of this laboratory.

RESULTS

1. *Photosensitivity of JEV and free JEV-RNA in the presence of PF*

JEV and free JEV-RNA samples with or without PF were irradiated by a 20 watt fluorescent lamp as described in Materials and Methods, while some samples were kept in the dark as controls. After irradiation, the infectivity of JEV and free JEV-RNA and the HA activity were measured. The results are shown in Table 1.

The infectivities of JEV and free JEV-RNA are photosensitive in the presence of PF but HA activity shows no decrease in titer. This photoinactivation reaction requires the simultaneous presence of dye and visible light, and addition of pre-irradiated dye to JEV caused no inactivation of JEV. Moreover, when pre-irradiated JEV was mixed with PF in the dark there was no decrease in PFU (not shown in the Table).

The kinetics of the photoinactivation give a single hit curve for JEV and for free JEV-RNA, indicating that one of photon-produced legions may be responsible for destroying the infectivity of a sensitized virus particle (Fig. 1).

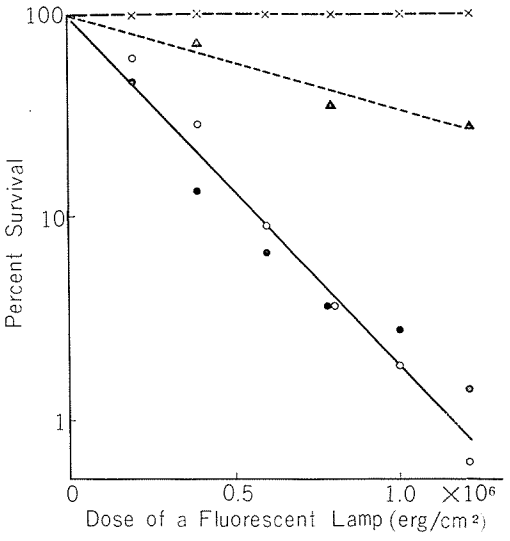

FIGURE 1 Photoinactivation of JEV and free JEV-RNA in the presence of proflavine (PF).
○——○ PFU of JEV
●——● PFU of RNA from irradiated JEV
▲------▲ PFU of free JEV-RNA
×——×——× HAU of JEV
PF: 10 μg/ml

TABLE 1 *The photosensitivity of JEV and free JEV-RNA in the presence of proflavine (PF)*

PF	Irradiation	Virus activity		Free RNA infectivity (log PFU/ml)
		(log PFU/ml)	(HAU/ml)	
+	+	4.13	1024	3.49
+	−	7.95	1024	4.60
−	+	7.90	1024	4.54
−	−	7.79	1024	4.41

JEV: U_2, PF: 10 μg/ml, Irradiation for 5 min.

To test whether photoinactivation damaged the RNA moiety of the virus, phenol extracts from samples of photoinactivated JEV which had received various energy doses during irradiation were assayed for infectious RNA. The results are also shown in Fig. 1. The curve representing the infectivities of phenolic RNA from photoinactivated JEV, is parallel with the curve of virus photoinactivation, suggesting that infectious RNA cannot be recovered from photoinactivated JEV and the major cause of the photodynamic inactivation of JEV is damage on the RNA moiety. When PF was mixed with yeast RNA or the JEV-RNA fraction, the fluorescence of PF was quenched but with the JEV fraction this change was not observed. The difference in the rate of the photoinactivation of JEV and free JEV-RNA seen in Table 1 and Fig. 1 is discussed later.

2. *Conditions affecting the rate of photoinactivation*

a. The effect of the PF concentration

S_1 samples of JEV diluted 100 fold with TVS and containing 0, 0.01, 0.1, 1.0, 10, and 100 μg/ml of PF respectively, were irradiated for 5 minutes by a fluorescent lamp. At a PF concentration of 1 μg/ml, infectivity was reduced most, namely by a factor of about 3 log units. At 100 and 0.01 μg/ml of PF, no significant reduction of PFU occurred as in the case of the dye free control and that kept in the dark. PF was used at a concentration of 1–10 μg/ml in subsequent experiments.

b. The effect of oxygen

It is well known that molecular oxygen is essential for the photoinactivation reaction. This was also found to be true in the case of JEV photoinactivation. As shown in Table 2, the rate of photoinactivation of JEV is suppressed when the air phase of the sample is replaced with N_2, and flushing of air into the sample again permits the reduction of infectivity by irradiation as in the case with untreated controls.

TABLE 2 *The effect of oxygen*

Atmosphere	Flushing with air	Irradiation	Virus infectivity (log PFU/ml)
N_2	−	−	5.04
N_2	−	+	4.58
N_2	+	−	4.18
N_2	+	+	<0.4
air	−	−	4.85
air	−	+	<0.4

JEV : 100 fold diluted S_1, PF : 1 μg/ml, Irradiation for 10 min.

TABLE 3 *The effect of the temperature during irradiation*

Irradiation with PF at :	Virus infectivity (log PFU/ml)
4°C	3.96
R. T. (ca. 25°C)	4.04
37°C	3.92
Original sample	5.04

JEV : 100 fold diluted S_1, PF : 10 μg/ml, Irradiation for 5 min.

TABLE 4 *The effect of the preincubation time with PF*

Sample	Preincubation time	Virus infectivity (log PFU/ml)
A	20 hr PF (+)	4.30
B	20 hr PF (−)	6.28
C	30 min PF (+)	4.00
D	30 min PF (−)	6.00
E	0 min PF (+)	4.08
F	0 min PF (−)	6.15

JEV : 10 fold diluted U_2, PF : 1 μg/ml, Irradiation for 5 min.

c. The effect of temperature

Table 3 shows that during irradiation no appreciable effect of temperature (4°C–37°C) on the photoinactivation rate is observed.

3. *The binding of PF by JEV*

a. Rapid binding of PF by JEV

To obtain the time course of the binding of PF by JEV, the following experiments were carried out. Duplicate JEV samples, one with and the other without PF, were preincubated in the dark at 4°C for various times before irradiation and then all samples were exposed to light under similar conditions. The final infectivities of the samples were titrated and the results are shown in Table 4. The photoinactivation rate is not affected by the time of preincubation of JEV with PF before irradiation (0 min.–20 hrs), indicating that the dye may be rapidly bound by JEV.

b. The reversibility of the binding of PF by JEV

The photosensitive mixture of PF and JEV was filtered through a Sephadex gel column in the dark and the photosensitivity of the fraction of the filtrate containing JEV was tested. As shown in Table 5, the JEV fraction of the filtrate had lost its sensitivity to visible light and re-addition of dye to this frac-

TABLE 5 *Reversibility of the binding of PF by JEV*

Sample	Sephadex gell filtration	Re-addition of PF	Irradiation	Virus activity	
				(log PFU/ml)	(HAU/ml)
a	−	−	−	6.78	512
b	−	−	+	4.61	512
c	+	−	−	6.08	32
d	+	−	+	5.97	32
e	+	+	−	5.85	32
f	+	+	+	3.25	32

JEV : 10 fold diluted S_1, PF : 2 μg/ml, Irradiation for 5 min.

TABLE 6 *The photosensitivity of the infectious RNA extracted from a not-irradiated PF-JEV mixture*

Sample for RNA extraction	Re-addition of PF after extraction	Irradiation	RNA infectivity (log PFU/ml)
JEV with PF	−	+	4.87
	−	−	4.76
	+	+	3.53
	+	−	4.53
JEV without PF	−	+	4.32
	−	−	4.00
	+	+	3.26
	+	−	4.42

The infectious RNA samples were extracted in darkness from not-irradiated JEV-S_1 with or without 10 μg/ml of PF and their photosensitivities were tested in the absence and presence of PF by irradiation for 5 min.

tion again caused reduction of infectivity by irradiation as in the case of the unfiltered controls. This result indicates that reversible dissociation of dyes from the photosensitive virus-dye complex occurs, rendering the virus photoresistant. The photoinactivated JEV could not be reactivated by Sephadex gell filtration (not shown in the Table). A similar reversibility was found with infectious RNA as shown in Table 6. The infectious RNA extracted in darkness from the photosensitive PF-JEV mixture was also photoresistant and the re-addition of PF to the extracted JEV-RNA again caused reduction of PFU by irradiation. These results seem to indicate that the binding of PF by JEV is reversible.

4. *The site of dye-sensitized photoinactivation of JEV*

To examine the site of dye-sensitized photoinactivation of JEV by PF, the following experiments were carried out. The visible absorption spectrum of free PF had a peak at 445 mμ. A shift of this peak toward longer wavelengths occurred on addition of yeast RNA, while no shift of the peak was observed on addition of bovine plasma albumin to PF. The action spectrum for JEV photoinactivation by PF, which was experessed in terms of the reciprocal of the inactivation dose to 37% survival as previously described (KONDO and JAGGER, 1966), was obtained using various monochromatic light beams, as described in Materials and Methods. As shown in Fig. 2, the action spectrum was close to the absorption spectrum of a mixture of PF and yeast RNA and significantly different from that of free PF. These results suggest that the dye changes its absorption peak by binding with the JEV-RNA moiety and that photon absorption by the RNA-PF complex gives rise to lethal damage on the viral RNA. This seems to coincide with the result that infectious RNA cannot be recovered from the photoinactivated JEV by the cold phenol method, as already described above.

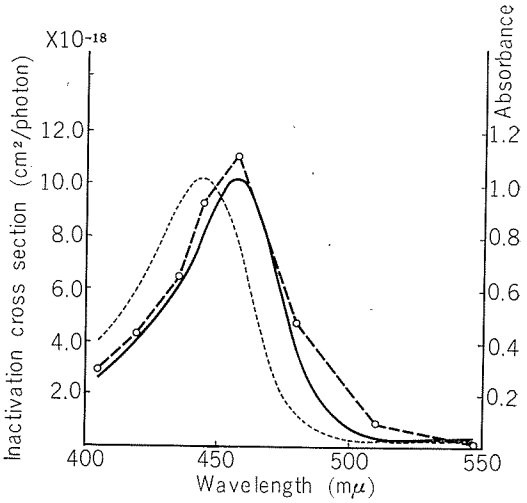

FIGURE 2 Action spectrum for photoinactivation of PF-JEV and absorption spectra of PF and PF-yeast RNA.
○----○ Action spectrum for the inactivation of PF-JEV
--------- Absorption spectrum of PF (10 μg/ml)
———— Absorption spectsum of PF (10 μg/ml) and yeast RNA (1.25 mg/ml) mixture
Medium : 0.02 M Tris-HCl (pH 7.6), 0.1 M NaCl

TABLE 7A *pH dependency of HA activity of photoinactivated JEV*

pH of VAD	HAU/0.5 ml of :	
	Original sample	Photoinactivated sample
5.8	<2	<2
6.0	80	80
6.2	160	160
6.4	40	80
6.6	<2	<2
6.8	<2	<2
7.0	<2	<2

TABLE 7B *HI test of photoinactivated JEV*

pH of VAD	HI titer of antiserum by :	
	Original sample	Photoinactivated sample
6.2	2560	2560
6.4	2560	2560

5. *Some characters of the photoinactivated JEV*

As aforementioned, the titer of the HA activity of JEV is not reduced by PF and visible light. The optimal pH for HA activity and the antigenicity against HI test of the photoinactivated JEV were examined. The results are shown in Table 7A and 7B. There was no appreciable difference between untreated and photoinactivated JEV in the optimal pH or the antigenicity against HI. The HA activity of the photoinactivated JEV also showed the same sedimentation pattern in a sucrose density gradient as that of untreated JEV, indicating that photoinactivation entails no gross change in the size of the JEV particle.

6. *Comparison of heat-inactivation and photoinactivation of JEV*

In Table 8, the characteristics of these two kinds of inactivation of JEV are compared. Mild heat-inactivation destroys the infectivity and HA activity of JEV but infectious RNA is recoverable from heat-inactivated JEV. Photoinactivation also reduces the infectivity but entails no reduction of HA activity of JEV and no infectious RNA can be recovered from the photoinactivated JEV.

TABLE 8 *Comparison of the heat-inactivation and photoinactivation of JEV*

Sample	Infectivity (PFU/ml) of:		V/R*	HAU/ml
	virus	extracted RNA		
Original	2.5×10^{7}	8.7×10^{3}	3.0×10^{3}	256
60°C, 4 min.	4.8×10^{2}	5.9×10^{3}	8.0×10^{-2}	<2
60°C, 8 min.	1.2×10^{2}	1.3×10^{3}	1.0×10^{-1}	<2
PF 2 μg/ml	5.0×10	$<5.0\times10$	>1.0	256
PF 10 μg/ml	2.5×10	$<5.0\times10$	>1.0	256

Irradiation for 8 min. *ratio=(virus PFU/RNA PFU)

DISCUSSION

The results described above indicate that the photoinactivation of JEV by PF is due to the inactivation of the JEV-RNA moiety rather than to loss of biological activities in the outer coat of the virus, in contrast with the case in heat-inactivation. Photon energy at 458 mμ, at which the monochromatic light beam inactivates the infectivity of JEV with PF most intensively, is estimated at 62.5 Kcal/mol and this value approximates to the average bond energy of C-N. Therefore, deamination of the RNA base may well cause the inactivation.

A difference in the rates of photoinactivation of JEV and free JEV-RNA was observed (Table 1 and Fig. 1). It should, however, be noted that the samples of JEV and JEV-RNA employed were not highly purified preparations because of difficulties in their purifications. When the JEV-S_1 fraction was diluted 10 fold with the RNA fraction, the photoinactivation rate of JEV was appreciably lower than that of a control diluted with buffer. This suggests that cellular RNAs which are present in excess in the RNA fraction may have a protective effect on the photoinactivation of JEV by PF. On the other hand, it is possible that the photoinactivation rate of JEV is really higher than that of free JEV-RNA and the difference in their rates of inactivation may reflect a difference in the configuration, or the degree of coiling of the helical structure, of JEV-RNA in the states of intra- and extra-particles which results in a difference in affinity to PF. As highly purified JEV and JEV-RNA samples were not obtained,

it was not possible to see whether the rates of both photoinactivations are really similar.

With regard to photoinactivation of viral RNA, only two instances have been reported: namely tobacco mosaic virus RNA is inactivated with acridine orange (CHESSIN, 1960) and poliovirus RNA is inactivated with toluidine blue (HIATT, 1960). Independently SCHAFFFER also observed photoinactivation of poliovirus RNA in the presence of PF (SCHAFFER, 1962). However, WILSON and COOPER failed to show any photosensitivity of poliovirus RNA with neutral red (WILSON and COOPER, 1965). They suggested that the discrepancy between these results and those of HIATT, might have been caused by use of a different molarity of salt solution.

The action spectrum for poliovirus photoinactivation by PF showed a slight shift toward longer wavelengths relative to the absorption, but the data were not sufficiently precise to be certain that this was a real phenomenon (SCHAFFER, 1962). On photoinactivation of JEV by PF, the action spectrum showed a significant shift and corresponded approximately to the absorption spectrum of a mixture of PF and yeast RNA. We were unable to obtain the action spectrum for free JEV-RNA photoinactivation by PF because of the impurity and relatively low titer in the infectivity of the JEV-RNA fraction.

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