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PREPARATION OF NON-INFECTIOUS ARBOVIRUS ANTIGENS BY PHOTO-DYNAMIC INACTIVATION

THE inactivation of viruses by different dyes in the presence of light has been known for a long time.[1] Viruses thus inactivated retain their antigenicity and are able to produce immunity in animals.[2-4] Like other viruses, arboviruses were also found to be susceptible to photodynamic inactivation by neutral red.[5] In view of the possibility of the laboratory workers contracting virus infections from infected antigens it was thought worthwhile to explore the possibilities of preparing non-infectious antigens by photodynamic inactivation. The present communication reports the results of the experiments carried out in this respect.

The viruses employed in the study were Japanese encephalitis (JE) P 20778 strain and the Kyasanur Forest disease virus (KFD) P 9605 strain. Stocks were prepared from infected suckling mouse brains which were suspended in phosphate saline containing 0·75% bovalbumin, centrifuged at 12,100 g. for one hour and stored at — 55° C.

Stock dye solutions were made in distilled water at 0·1% concentrations and sterilized by autoclaving at 10 lbs. pressure for 10 minutes. The following dyes were employed: methylene blue (Grubler and Co., Leipzig), brilliant cresyl blue (E. Merck A.G., Darmstadt), malachite green (B.D.H., England,) Acriflavin (Western Pharma. Works, Bombay) and neutral red (National Aniline, U.S.A.).

Antigens were prepared from infected suckling mouse brains by the acetone-ether method of Clarke and Casals.[6] In some experiments saline extracted antigens treated with protamine sulphate were also employed.

Stock virus suspensions were diluted in phosphate buffered saline (pH 7·6) or in BAPS (pH 7·2) and transferred to a petri dish. The fluid depth was usually kept about 2 mm. Appropriate amounts of the dyes were added to the virus suspension so as to give 1 : 100,000 concentration of the dye in the reaction mixture. Light from a 100 watt microscope lamp filtered through a ground glass filter was allowed to fall upon the reaction mixture. In all experiments 400 foot candles of illumination was employed. Aliquot samples of 0·8 ml. were taken out at different intervals in test-tubes coated with black paint on the outside. Controls of unilluminated reaction mixtures (controls kept in darkness) with dye omitted (controls kept in light) were included in all the experiments. The reactions were carried out at 4° C. in a cold room.

The antigens were subjected to photoinactivation with cresyl blue under identical conditions.

Infectivity assays were made by intracerebral inoculations in mice. Haemagglutination tests (HA) were carried out by the method of Clarke and Casals,[6] and complement fixation (CF) tests by the method described by Pavri *et al.*[7]

Preliminary screening for the capability of the different dyes of inactivating the viruses in the presence of light was done. Brilliant cresyl blue, methylene blue and neutral red were found to be effective. The remaining of the dyes were either inactive or very weakly active.

The kinetics of virus inactivation was studied with cresyl blue and neutral red. When the $\log_{10}$ of the residual virus was plotted against the time of exposure to light, a straight line was obtained (Fig. 1) thus indicating a reaction of the first degree. The slope of the line obtained with cresyl blue was more steep than that with neutral red, thus indicating the faster rate of inactivation with cresyl blue. Though no kinetic studies were done with methylene blue, it appeared from the preliminary experiments that it inactivated

approximately the same amount of virus in about the same time as was with cresyl blue.

When the antigens prepared by acetone-ether extraction method were photoinactivated with cresyl blue, they were rendered non-infectious without any significant alteration in the HA and CF titres. The saline extracted protamine sulphate treated antigens behaved likewise.

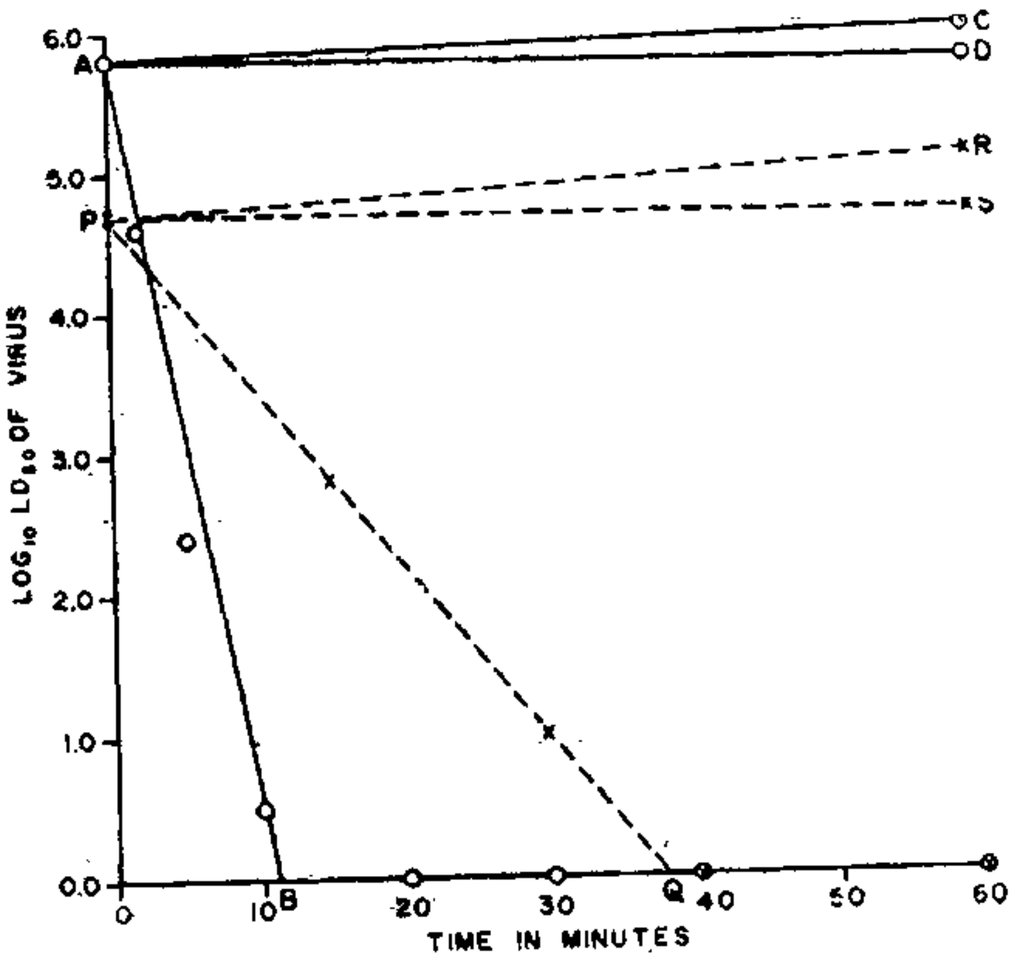

FIG. 1.- Inactivation of J E virus with dyes.

AB	..	Inactivation with cresyl blue.
AC	..	Control with cresyl blue kept in darkness.
AD	..	Control without cresyl blue in light.
PQ	..	Inactivation with neutral red.
PR	..	Control with neutral red kept in darkness.
PS	..	Control without neutral red in light.

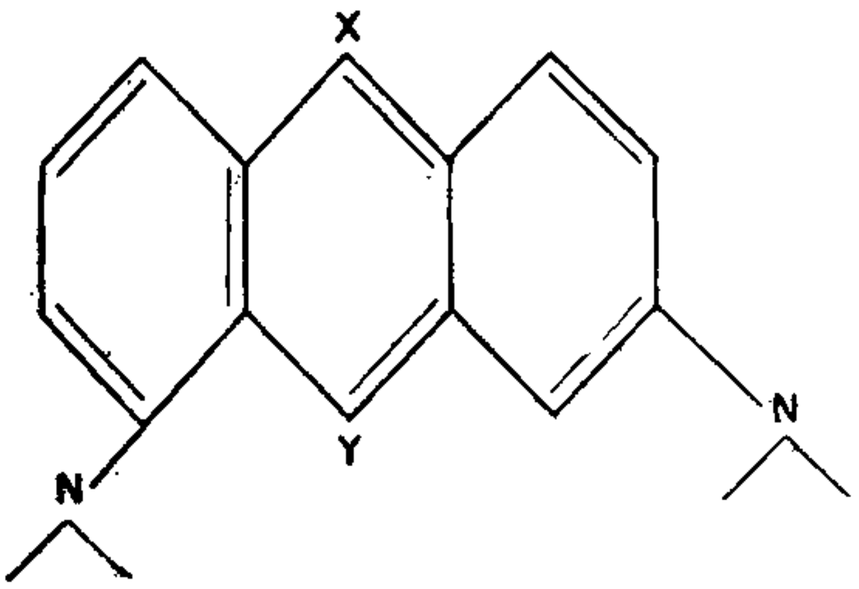

FIG. 2. General formula of photo-inactivating dyes.

From the above results it appears that cresyl blue is eminently suitable for photoinactivation of JE and KFD viruses. Methylene blue seems to be almost equally effective in producing photoinactivation. Crowther and Melnick[8] while considering the structures of the photodynamically active compounds, *viz.*, neutral red, toluidine blue and acridine orange, showed close resemblance in chemical structure among them. Figure 2 shows the general formula of the photoactive dyes. In the case of methylene blue the positions 'X' and 'Y' are occupied by nitrogen and sulphur respectively; in the case of cresyl blue by nitrogen and oxygen; and in the case of neutral red both the positions are occupied by nitrogen atoms. In acriflavin the position 'X' is occupied by a carbon atom and the position 'Y' by a pentavalent nitrogen. It was interesting to note that the strongly basic quinone-imine dye cresyl blue showed a faster rate of photoinactivation than the weakly basic neutral red also a quinone-imine dye. Acriflavin though a basic dye, belonging to the xanthene group was very weakly active. Malachite green which is a triphenyl methane dye was completely ineffective.

The photodynamically inactivated antigens do not show significant variations in titres from the untreated antigens in HA or in CF. Therefore this seems to be a simple and convenient method of preparing non-infectious antigens.

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ON THE OCCURRENCE OF SOME TRACE FOSSILS IN THE BHANDER LIMESTONE (UPPER VINDHYAN) OF REWA DISTRICT, M.P.*

THE present paper records the occurrence of *trace fossils* in the Bhander Limestone Series of the Upper Vindhyans in the Bankuiyan (24° 01' : 81° 12'; 63 H/NW) area, Rewa District, Madhya Pradesh. The fossils dealt with in this paper consist of tracks and trails,