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Sensitivity of bluetongue virus to lipid solvents, trypsin and pH changes and its serological relationship to arboviruses*

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Arboviruses are considered to have the following properties in common: they contain RNA and are sensitive to lipid solvents, biologic transmission by a vector is obligatory and they are pathogenic for Swiss mice by the intracerebral route. There is cogent evidence that bluetongue virus (BTV) is transmitted by arthropods (Du Toit, 1944; Foster, Jones & McCrory, 1963; Price & Hardy, 1954) and biological transmission is probable (Foster *et al.* 1963). The nucleic acid of BTV is believed to be RNA (Livingstone & Moore, 1962; Svehag, 1963) and the virus is pathogenic for mice by the intracerebral route (Svehag, 1962). For these reasons BTV has been tacitly classified as an arbovirus. However, BTV has not been placed in any of the described serological groups of arboviruses (Andrewes, 1964; Casals, 1961). Further, BTV possesses two characteristics which are unique for members of the arbovirus group, viz. its recently reported insensitivity to ether treatment (Studdert, 1965) and the fact that it is transmitted by gnats of the genus *Culicoides* (Diptera: *Ceratopogonidae*). This latter property it shares with African horse sickness virus which, like BTV is classified among 'ungrouped' arboviruses (Casals, 1961).

As physical and chemical properties in combination with antigenic relationships serve as more precise criteria for virus classification than pathogenicity and tissue affinity, the sensitivity of BTV to various chemical treatments was studied. The stability of BTV to lipid solvents such as ether or sodium deoxycholate (SDC) was confirmed and this observation extended to chloroform, a solvent of greater polarity. Cheng (1958) reported that trypsin inactivated arboviruses of the serological group B, while viruses in group A were unaffected. As this finding suggested that treatment with proteases can aid in the classification of arboviruses, the effect of trypsin on BTV was examined. The utility of exposure to low pH for the classi-

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fication of virus strains is well illustrated by the picorna virus group, all enteroviruses being resistant to low pH while rhinoviruses rapidly lose their infectivity below pH 5·0–6·0. This treatment, useful as a preliminary step in virus identification, was also applied to BTV.

MATERIALS AND METHODS

Virus

A strain (BT-8) of bluetongue virus recovered from infected sheep in California (McKercher, McGowan & Saito, 1954) and originally passaged by yolk sac inoculation of chicken embryos was employed. BT-8 at the 180th embryo passage was adapted to the intracerebral route in unweaned mice (Svehag, 1962). Stock virus, at the 53rd mouse passage, was prepared in Tris buffer, pH 7·3 containing 200 i.u. penicillin and 100 μg. streptomycin per ml. The suspension was clarified by centrifugation at 3000 rev./min. for 20 min., pelleted by centrifugation at 30,000 rev./min. for 3 hr. and the pellet dissolved in Tris buffer and stored at $-70°$ C. in sealed glass ampoules.

The 65th passage of the Wisconsin FXNO strain of distemper virus (DV) was propagated in chicken embryos by the stab method (Gorham, 1957). Stock virus was prepared from infected chorioallantoic membranes in phosphate buffer, pH 7·4 containing 5% glycerine, and 50 μg. dihydrostreptomycin sulphate and 200 i.u. penicillin G potassium per ml. The suspension was clarified by low speed centrifugation and the virus stock stored at $-70°$ C. in sealed glass ampoules.

Infectivity assay

BTV was serially diluted five-fold or ten-fold and assayed intracerebrally (0·02 ml.) in 3-day-old unweaned Webster Swiss albino mice using 7–8 mice per dilution. Virus titres were expressed in $\log_{10}$ MLD 50 per ml. undiluted mouse brain homogenate. DV was titrated in embryonated eggs by the stab method (0·2 ml.), using five-fold dilutions and five chorioallantoic membranes per dilution. Virus titres were expressed in $\log_{10}$ EID 50 per ml. undiluted chorioallantoic membrane homogenate.

Neutralization tests

Two thousand LD 50 (0·2 ml.) of virus was mixed with 1·8 ml. antiserum; the reaction mixture was shaken and incubated at 37° C. for 24 hr. After incubation the virus-serum mixture was placed in an ice bath, and titrated for residual infectivity using two-fold serial dilutions.

Treatment with lipid solvents

Two ml. BTV preparation and 0·5 ml. diethyl ether (20%) were placed in air-tight tubes, shaken and incubated at 5° C. for 24 hr. during which time the tubes were intermittently shaken. Virus controls devoid of ether were similarly treated. After incubation the ether was removed and residual ether allowed to evaporate. The samples were then diluted in Tris buffer and assayed for infectivity.

One ml. BTV or DV preparation and 0·05 ml. chloroform were intermittently

shaken for either 10 min. at room temperature, 10 min. at 5° C. or 24 hr. at 5° C. The mixtures and virus controls were then centrifuged at 400 rev./min. for 5 min. and the clear top phase recovered and titrated for infectivity.

One ml. BTV preparation and 1 ml. SDC (1/500) in Tris buffer, pH 7·3 were mixed and incubated for 1 or 2 hr. at 37° C. These mixtures and virus controls, in which the Tris buffer replaced deoxycholate, were then assayed for infectivity.

Treatment with trypsin

Two different trypsin preparations were used: Difco Bacto trypsin (1/250) once crystallized and a trypsin preparation twice crystallized and salt free (Worthington Biochemical Corp., Freehold, New Jersey). The concentration of trypsin used varied between 0·05 and 20 mg./ml. The soybean trypsin inhibitor, 5 times crystallized (Worthington Biochemical Corp.) was employed at a concentration of 8 mg./ml. Both trypsin and the soybean trypsin inhibitor were dissolved in phosphate buffer, pH 8·0. Fresh trypsin solutions were prepared before each test since the trypsin gradually lost its activity in phosphate buffer at 4° C. For details concerning the experimental design see legend to Fig. 3.

RESULTS

Sensitivity to lipid solvents

BTV was found to be rather resistant to treatment with both ether, chloroform and SDC (Table 1). Treatment with chloroform was preferred to ether as the chloroform could be readily separated from the virus suspension by low-speed centrifugation while the ether had to be removed by evaporation. It appeared to be immaterial whether the virus preparation was incubated and shaken with chloroform for 10 min. or 24 hr. DV was used as a chloroform sensitive control virus.

Table 1. *Effect of lipid solvents on bluetongue virus*

Experiment	Incubation conditions	Treated	Control	No tests
BTV* + 0·1 % SDC	1 hr., 37° C.	7·0†	7·3	5
	2 hr., 37° C.	7·0	7·6	1
BTV‡ + ethyl ether	24 hr., 5° C.	8·0	8·3	3
BTV‡ + chloroform	10 min., 5° C.	7·0	7·6	2
	10 min., room temp.	7·4	8·1	2
	24 hr., 5° C.	7·5	8·3	1
DV‡ + chloroform	10 min., room temp.	0	4·5	2

* Final virus dilution $10^{-1·0}$. † Expressed as $\log_{10}$ LD50/ml.
‡ Final virus dilution $10^{-0·7}$.

Sensitivity to pH *changes*

The data in Fig. 1 show that BTV had a narrow zone of pH stability in Michaelis buffer between pH 6 and 8 similar to that of rhinoviruses and foot-and-mouth disease virus which also are RNA-containing and ether-resistant viruses. This is in contrast to most picornaviruses which are stable between pH 2 and 9. Reoviruses are reported to be stable between pH 4 and 8 (Wallis, Smith & Melnick, 1964).

Like several arboviruses (Western equine encephalomyelitis (WEE), Japanese B encephalitis, St Louis encephalitis), BTV was slightly more stable at alkaline than at acid pH. Eastern equine encephalomyelitis (EEE), Western equine encephalomyelitis and Semliki Forest virus resemble BTV also in their sensitivity to acid pH, EEE and WEE being inactivated below pH 6·4 and Semliki Forest virus below pH 6 (Andrewes, 1964).

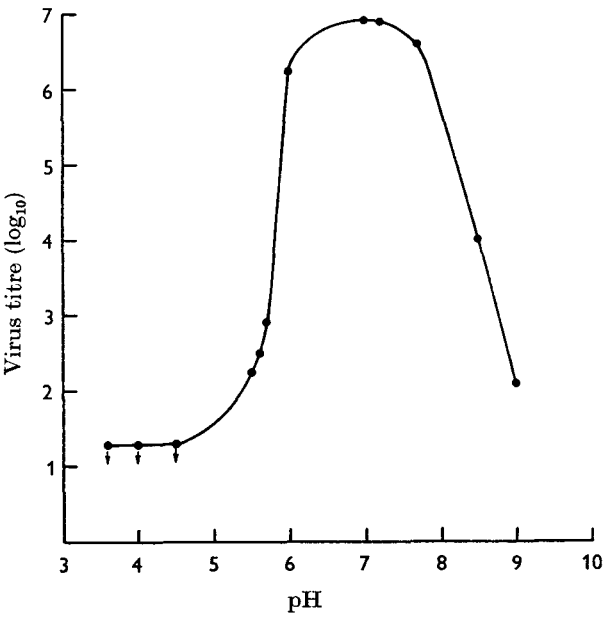

Fig. 1. Stability of BTV in Michaelis buffer at various pH values. 0·2 ml. BTV preparation (1/5) was mixed with 1·8 ml. Michaelis buffer and the pH of the mixture readjusted to neutrality with 0·1 N-NaOH after 1 min. incubation at 37 °C.; pH determinations were made on the BTV–buffer mixtures.

The inactivation of BTV within 1 min. below pH 6 and above pH 8 rendered it difficult to study the kinetics of the inactivation reaction in these pH regions. A pH (6·1) in the critical region was therefore chosen for a study of the reaction kinetics (Fig. 2). The kinetic curve had two components, about 90% of the virus being inactivated rapidly while the residual infectivity was lost at a much reduced rate. A minor fraction of the virus population ($< 0{\cdot}01\%$) was rather resistant at this pH. This may be due to a protective effect from the clumping of virus particles or from extraneous protein in the virus preparation.

The pH inactivation appeared to be irreversible as the degree of inactivation was the same whether treated virus was assayed for infectivity immediately or several hours after adjustment of pH to neutrality.

Sensitivity to trypsin treatment

In the present study BTV was found to be inactivated to about 90% by relatively low trypsin concentrations (50–100 μg/ml.) and the degree of inactivation to be proportional to the enzyme concentration (Fig. 3). The effect was not due to the

presence of nucleases or other contaminating active principles in the trypsin preparation as (1) two trypsin preparations of different purity gave identical results, and (2) the trypsin effect was completely abolished by preincubation of the trypsin with soybean trypsin inhibitor (8 mg./ml.), which is known to block only the active proteolytic part of the trypsin molecule (Green, 1953; Kunitz, 1947). It was also

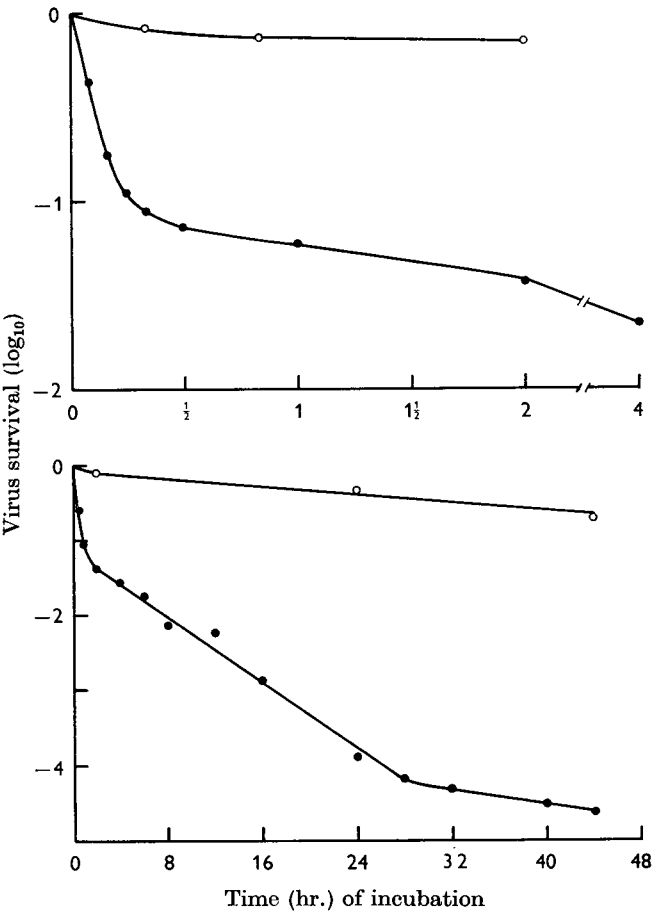

Fig. 2. Kinetics of the inactivation of BTV infectivity in Michaelis buffer at 37° C. 0·2 ml. BTV preparation (1/5) and 1·8 ml. Michaelis buffer, pH 6·1, ●; 0·2 ml. BTV preparation and 1·8 ml. Tris buffer, pH 7·3, ○; pH determinations were made on the BTV–buffer mixtures. The points represent the geometric means of two tests.

demonstrated that trypsin-inactivated BTV was not reactivated by the addition of the trypsin inhibitor. These results indicated that the inactivation occurred via proteolysis of the viral particle and not simply by binding to virus receptors. Soybean trypsin inhibitor alone did not inactivate BTV.

It was unlikely that the trypsin effect was mediated by degradation products of brain tissue as the degree of BTV inactivation was diminished rather than increased when crude BTV preparations were used.

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Serological classification

The grouping of arboviruses is based solely on immunological cross-reactions. The haemagglutination-inhibition test provides the broadest spectrum of antigenic overlap among these viruses, while the intracerebral neutralization test, in general, is more specific. Casals (1961) and Clarke (1960) have improved and extensively employed the former test for classification of arboviruses and Casals has divided arboviruses into four antigenic groups designated A, B, C and Bunyamwera groups. Remaining arboviruses are classified as 'ungrouped' and BTV is included in this group (Casals, 1961).

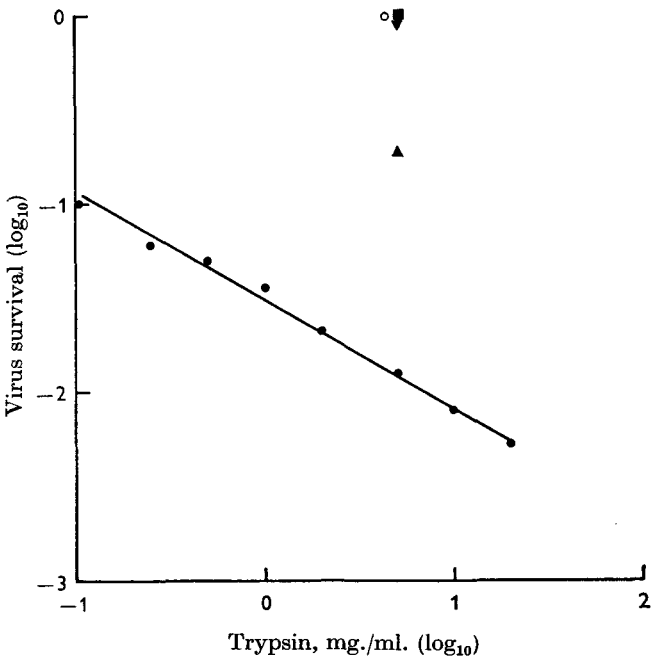

Fig. 3. Effect of trypsin and soybean trypsin inhibitor on BTV infectivity; 0·2 ml. BTV preparation (1/5) was mixed with either 1·8 ml. trypsin solution, ●; 1·8 ml. trypsin solution heated at 56° C. for 30 min., ▲; 1·8 ml. trypsin solution containing 15 mg. soybean trypsin inhibitor, ■; 1·8 ml. phosphate buffer containing 15 mg. soybean trypsin inhibitor, ▼, or 1·8 ml. phosphate buffer, pH 8·0, ○. All mixtures were incubated for 24 hr. at 37° C. before assay for infectivity. The points represent the geometric means of two to three tests.

The finding that BTV like arboviruses in group B was inactivated by trypsin led us to re-examine the possibility that an immunological cross-reaction existed between BTV and viruses belonging to this group. Virus neutralization, employing the immuno-inactivation technique (Gard, 1957) and the intracerebral route in unweaned mice was used. Immune mouse sera,* the lowest dilution tested being 1/10, prepared against St Louis, West Nile and Japanese B viruses (group B) were, however, found to exert no greater neutralizing effect on BTV than normal mouse

* Kindly supplied by Dr J. Casals, The Rockefeller Institute, New York.

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serum. Similar results were obtained when immune mouse sera produced with EEE-85, Sindbis, Chikungunya viruses (group A), Carparu and Oriboca viruses (groups C) and Bunyamwera, Chittoor and Illesha viruses (the Bunyamwera group) were assayed against BTV. It should be emphasized, however, that these results require confirmation with the hemagglutination-inhibition test because of its greater sensitivity for the detection of cross-reactions.

DISCUSSION

All arboviruses so far examined have been sensitive to ether or SDC and this property has served to distinguish them from viruses such as picorna- and adenoviruses. The sensitivity of arboviruses to lipid solvents and the finding that RNA can be extracted from these viruses only by the 'hot phenol' method (Wecker, 1959) suggest that lipids are contained in an envelope which protects the RNA. Electron micrographs of WEE virus (Morgan, Howe & Rose, 1961) and Sindbis virus (Mussgay & Rott, 1964) have also indicated the existence of such an envelope. Myxoviruses and viruses belonging to the herpes group, which also are sensitive to lipid solvents, are all known to possess an envelope. The stability of BTV to lipid solvents and its sensitivity to trypsin indicates that this virus, in contrast to other arboviruses, is lacking a lipid-containing envelope but has a surface protein which interacts with the host cell. In a recent communication, Studdert (1965) states that unpublished electron micrographs suggest that BTV is devoid of an envelope.

Determination of the effect of enzymic action may provide useful information for the classification of virus groups (Gresser & Enders, 1961). As early as 1936 Merrill reported that EEE virus, belonging to group A arboviruses, was resistant to trypsin, and Cheng (1958) recently found this property to be shared by all group A arboviruses, while in contrast group B arboviruses were digestible by trypsin. In the present study, BTV was found to be trypsin sensitive but attempts to demonstrate a serological relationship between this virus and representative group B arboviruses failed.

The present results do not support the inclusion of BTV in the arbovirus group. It is instead becoming evident that BTV shares many properties with reoviruses. Both viruses contain RNA, resist treatment with lipid solvents (Gomatos, Tamm, Dales & Franklin, 1962) and are trypsin sensitive (Gomatos & Tamm, 1962; Mayor & Jordan, 1965). Of interest in this context are results by Polson & Deeks (1963) indicating that African horse sickness virus, another serologically unclassified, RNA-containing and ether-resistant arbovirus, which also is unstable below pH 6 and transmitted by *Culicoides* spp., has an ultrastructure which closely resembles that of reoviruses (Vasques & Tournier, 1962; Jordan & Mayor, 1962).

SUMMARY

Bluetongue virus was found to be resistant to ether, chloroform and sodium deoxycholate under a variety of conditions but sensitive to treatment with trypsin. The virus had a narrow zone of pH stability between pH 6 and 8 in Michaelis

buffer. Below pH 6 bluetongue was irreversibly inactivated within 1 min. at 37° C. In many of its characteristics, bluetongue virus appears to be closely related to the reoviruses.

REFERENCES

Andrews, C. H. (1964). *Viruses of Vertebrates.* London: Baillière, Tindall and Cox.

Casals, J. (1961). Procedures for identification of arthropod-borne viruses. *Bull. Wld Hlth Org.* **24**, 723.

Cheng, P-Y. (1958). The inactivation of group B arthropod-borne animal viruses by proteases. *Virology* **6**, 129.

Clarke, D. H. (1960). Antigenic analysis of certain group B arthropod-borne viruses by antibody absorption. *J. exp. Med.* **111**, 21.

Du Toit, R. M. (1944). The transmission of blue-tongue and horse sickness by *Culicoides*. *Onderstepoort. J. vet. Sci. Anim. Ind.* **19**, 7.

Foster, N. M., Jones, R. H. & McCrory, B. R. (1963). Preliminary investigations on insect transmission of bluetongue virus in sheep. *Am. J. vet. Res.* **24**, 1195.

Gard, S. (1957). Immuno-inactivation of poliovirus. *Arch. ges. Virusforsch.* **7**, 449.

Gomatos, P. J. & Tamm, I. (1962). Reactive sites of reovirus type 3 and their interaction with receptor substances. *Virology* **17**, 455.

Gomatos, P. J., Tamm, I. Dales, S. & Franklin, R. M. (1962). Reovirus type 3: physical characteristics and interaction with L cells. *Virology* **17**, 441.

Gorham, J. R. (1957). A simple technique for the inoculation of the chorio-allantoic membrane of chicken embryos. *Am. J. vet. Res.* **18**, 691.

Green, N. M. (1953). Competition among trypsin inhibitors. *J. biol. Chem.* **205**, 535.

Gresser, I. & Enders, J. F. (1961). The effect of trypsin on representative myxoviruses. *Virology* **13**, 420.

Jordan, L. E. & Mayor, H. D. (1962). The fine structure of reovirus, a new member of the icosahedral series. *Virology* **17**, 597.

Kunitz, M. (1947). Crystalline soybean trypsin inhibitor. II. General properties. *J. gen. Physiol.* **30**, 291.

Livingstone, C. W. & Moore, R. W. (1962). Cytochemical changes of bluetongue virus in tissue cultures. *Am. J. vet. Res.* **23**, 701.

Mayor, H. D. & Jordan, L. E. (1965). Studies on reovirus. I. Morphologic observations on the development of reovirus in tissue culture. *Expl molec. Path.* **4**, 40.

McKercher, D. G., McGowan, B. & Saito, J. K. (1954). Studies on bluetongue. I. Isolation, identification and typing of the bluetongue virus and a preliminary report on the serodiagnosis of the disease. *Proc. Am. vet. med. Ass.* p. 167.

Merrill, M. H. (1936). Effect of purified enzymes on viruses and Gram-negative bacteria. *J. exp. Med.* **64**, 19.

Morgan, C., Howe, C. & Rose, H. M. (1961). Structure and development of viruses as observed in the electron microscope. V. Western equine encephalomyelitis virus. *J. exp. Med.* **113**, 219.

Mussgay, M. & Rott, R. (1964). Studies on the structure of a hemagglutinating component of a group A arbo virus (Sindbis). *Virology* **23**, 573.

Polson, A. & Deeks, D. (1963). Electron microscopy of neurotropic African horse-sickness virus. *J. Hyg., Camb.*, **61**, 149.

Price, D. A. & Hardy, W. T. (1954). Isolation of the bluetongue virus from Texas sheep—Culicoides shown to be a vector. *J. Am. vet. med. Ass.* **124**, 255.

Studdert, M. J. (1965). Sensitivity of bluetongue virus to ether and sodium deoxycholate. *Proc. Soc. exp. Biol. Med.* **118**, 1006.

Svehag, S-E. (1962). Quantitative studies of blue tongue virus in mice. *Arch. ges. Virusforsch.* **12**, 363.

Svehag, S-E. (1963). Thermal inactivation of blue tongue virus. *Arch. ges. Virusforsch.* **13**, 499.

Vasquez, C. & Tournier, P. (1962). The morphology of reovirus. *Virology* **17**, 503.

Wallis, C., Smith, K. O. & Melnick, J. L. (1964). Reovirus activation by heating and inactivation by cooling in $MgCl_2$ solutions. *Virology* **22**, 608.

Wecker, E. (1959). The extraction of infectious virus nucleic acid with hot phenol. *Virology* **7**, 241.