

HOG CHOLERA VIRUS : SENSITIVITY TO HYDROLYTIC ENZYMES *

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Résumé

VIRUS DE LA PESTE PORCINE CLASSIQUE : SENSIBILITE AUX ENZYMES HYDROLYTIQUES.
— L'action de la trypsine et de la phospholipase C sur l'infectiosité du virus de la Peste Porcine Classique a été étudiée. Les cinétiques d'inactivation montrent une baisse marquée du pouvoir infectieux en présence de ces deux enzymes. La sensibilité particulière de ce virus à un traitement protéolytique éclaircit certains aspects de son comportement sur le terrain. L'infectiosité du virus paraît dépendre de l'intégrité des phospholipides membranaires. Aucune corrélation n'a pu être établie entre le taux d'inactivation et le pouvoir pathogène des souches.

Introduction

Hog Cholera (HC), a viral disease specific to *suidae*, is today a major preoccupation of porcine husbandry. Only slow progress is being made in the study of the causal agent, due to numerous problems of handling of the virus in the laboratory.

Although information on the biochemical nature of the virus is still highly incomplete, it can nevertheless be classified in the *Togaviridae* family (Horzinek *et al.*, 1971). HCV is a small enveloped RNA-containing virus (Dinter, 1963; Loan, 1964; Kubin, 1967; Horzinek *et al.*, 1967; Mayr *et al.*, 1967; McKissick and Gustafson, 1967; Scherrer *et al.*, 1970; Laude, 1974).

The HCV exhibits a rather stable infectivity under natural conditions. It is not very sensitive to the actions of heat (Vittoz, 1961; Savi *et al.*, 1965; Helwig and Keast, 1966; Kubin, 1967), freezing (Vittoz, 1961; Popa *et al.*, 1963; Fuchs, 1968), dessication (Verge and Goret, 1941; Alboiu *et al.*, 1962; Schmitt-diel and Beck, 1968) and pH changes (Gheorghiu *et al.*, 1960). It seems however to exhibit a weak resistance to putrefying agents present in liquid manure (Jackson and Cabot, 1930) or in decomposing organs (David, 1931 and Hegyeli, 1936 cited by Verge and Goret, 1953).

Numerous investigations have been performed concerning the resistance of the HCV to chemical inactivation (review : Mahnel and

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Mayr, 1974), but information related to decreases in its infectivity by enzymes remains fragmentary and controversial. Thus, its sensitivity to trypsin is still ill-defined: whereas Van Bekkum and Barteling (1970) found HCV to be very sensitive, Matheka *et al.* (1962), Dinter (1963) reported only moderate sensitivity and Kubin (1967) none at all. In addition, an increase in its infectivity has also been reported following trypsin treatment (Collins, 1960).

Previous data concerning the comparative resistance of different strains to heat and various pH have shown that only a heat treatment makes easily feasible a distinction between pathogenic and attenuated strains (Aynaud, 1972).

The present report on a fully virulent and an attenuated strain was intended to furnish new data defining in greater detail the behavior of the HCV towards two hydrolytic enzymes, trypsin and phospholipase C.

Materials and methods

1. — *Viruses and cells*

The origin of the virus strains used has been reported previously (Aynaud *et al.*, 1972; Corthier *et al.*, 1974). The Alfort clone was isolated from a wild-type strain highly pathogenic for pigs. The Thiverval clone is an attenuated mutant, isolated from the Alfort clone.

Virus stocks were prepared in Pk₁₅ cells as described previously (Aynaud *et al.*, 1972). Infected cells were grown in Eagle's medium lacking calcium and supplemented with 5% foetal calf serum. After incubation, the cultures were frozen at -20°C in the presence of 5% dimethylsulfoxide. The solutions were clarified by centrifugation at 7,000 g for 20 min just prior to use.

2. — *Virus titration*

Virus infectivity was measured by a method of direct immunofluorescence on a monolayer Pk₁₅ (Cabrey *et al.*, 1965), previously described by Aynaud (1968).

3. — *Enzymatic treatments*

A suspension of each strain was diluted 1/10 in an enzyme solution of appropriate concentration, prepared by dissolving the enzyme in Eagle's medium, pH 7.6. The reaction mixture was immediately placed in a 37°C water bath and samples were withdrawn at regular intervals. The reaction was stopped by the addition of inhibitor followed

by transfer to ice-water.

Crystalline, B grade trypsin (Calbiochem) was utilized. Soybean trypsin inhibitor (m.w. 21500) [Nutr. Bioch. Corp.], was added to the reaction mixture at a concentration double that of the enzyme.

Clostridium perfringens phospholipase C was partially purified (Worthington Bioch. Corp., 1.5 U/mg). The inhibitor employed was EDTA, used at 1.5 mM per unit phospholipase (Macchia and Pastan, 1967). In order to detect any contaminating proteolytic activity in the commercial phospholipase preparations, carboxymethyl-lysozyme, as test material, was incubated with and without phospholipase and UV monitored after chromatography on Sephadex G-50 columns (Pharmacia). The appearance of two additional peptides in the treated samples showed existence of a slightly contaminating protease (~ 10%).

4. — *Measurement of the effects of enzymatic treatments*

After blocking of enzyme action, residual infectivity in various samples was titrated. In order to have an idea of the state of degradation of the particles after trypsin and phospholipase action, the following treatments were performed:

- ribonuclease A (Worthington), 20 µg/ml at 37°C for 60 min;
- seroneutralization with 0.2% anti-Thiverval serum for 60 min at 37°C;
- diethylaminoethyl-dextran: during titration, the sample was diluted in Eagle's medium containing varying concentrations of DEAE-dextran (m.w. 2×10^6).

Results

1. — *Trypsin action*

● In a preliminary experiment with the Alfort strain, we sought to define the influence of enzyme concentration (Fig. 1). A pronounced decrease in infectivity was recorded after 60 min at 37°C: residual infectivity was 1% at 100 µg/ml and 0.01% at 500 µg/ml, an inactivation plateau being practically reached at the latter concentration.

Inactivation kinetics were obtained with these two concentrations, using the Alfort and Thiverval strains. The resulting curves have similar shapes (Fig. 2). The decrease in infectivity at 100 µg/ml is represented by a first order reaction curve. At a concentration of 500 µg/ml, the reaction apparently is of a more complex order. Indeed, it can be interpreted as two successive first order

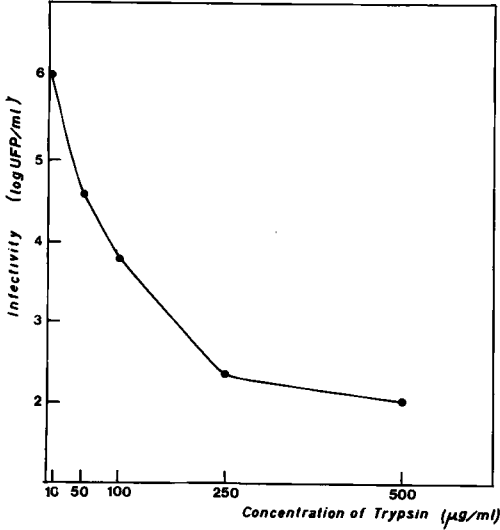


Fig. 1. HCV (Alfort strain) inactivation as a function of the trypsin concentration. Incubation was for 60 min at 37 °C.

TABLE 1

Treatment	Residual infectivity (%)
None	99
Tryps. Inhibitor	110
Trypsin	0.012
Trypsin + inhibitor	95
Trypsin + 10 x protein *	0.014

Table 1. Effect of trypsin on the infectivity of HCV after an incubation at 37 °C for 60 min. Trypsin : 500 µg/ml ; soybean trypsin inhibitor : 1 000 µg/ml (added at the beginning of the incubation). Enrichment of the reaction mixture in proteins is done by diluting the enzyme 1/10 in the virulent suspension (*).

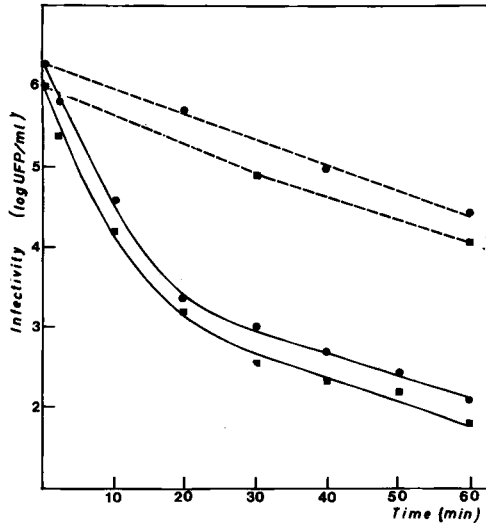


Fig. 2. Inactivation kinetics of HCV at 37 °C in the presence of 100 (---) or 500 (—) µg/ml trypsin. Strains Alfort (●) or Thiverval (■).

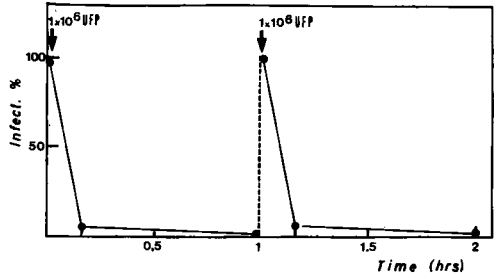


Fig. 3. Effect of trypsin (500 µg/ml at 37 °C) on the HCV infectivity. One hour after the beginning of the incubation, the infectious titer was brought again to its initial level.

reactions which are characterized by different rate constants.

● The results of different assays relative to a definition of the mode of action of trypsin in our experiments are summarized in Table 1 and Fig. 3. The infectious titer of untreated

virus during the reaction is modified to any appreciable extent by an incubation at 37 °C or by a non-specific effect of the trypsin inhibitor itself. The addition of foreign proteins does not change inactivation rate, an observation which renders doubtful a possible inactivation by a degradation product created in the complex reaction mixture. In addition, the reaction is 95 % inhibited by the soybean protein, which is highly specific for the proteolytic site of the enzyme (Green, 1953). Lastly, enzyme activity is not modified by an initial round of inactivation (Fig. 3). The sum of these results suggests that HCV inactivation is the result of a direct proteolytic action on the virus particle itself, consistent with

TABLE 2

Treatment (60 mn — 37 °C)	Residual infectivity (%)	
	Non-trypsinized sample	Trypsinized sample
Ribonuclease (10 µg/ml)	90	92
Anti HCV serum (0.2 %)	1.2 S.I. * = 1.99	2.5 S.I. = 2.07
DEAE-dextran (µg/ml)		
10	90	83
30	120	92
75	110	125
100	80	180
250 **	80	66
500 **	50	50

* : S.I. : seroneutralisation index.

** : this dose is slightly cytotoxic.

Table 2. Effects of various treatments on the infectivity (Alfort strain) of the trypsinized virus (500 µg/ml trypsin for 60 min at 37 °C).

previous observations on Arboviruses (Cheng, 1958).

● Using various experimental approaches, we tried to estimate the eventual degree of degradation of the residual infectious virus after enzymatic treatment : degree of RNA protection (Ribonuclease) and persistence of surface antigens (seroneutralization). We also examined the effect of DEAE-dextran on the charge state of the particle : this polycation is often utilized to stimulate the infectivity of viral suspensions, degraded or not (Pagano, 1970 ; Vaheri *et al.*, 1967). The results (Table 2) suggest that the infectivity of the trypsin-treated and control virus particles responds similarly to these various treatments, indicating that the residual virus has not at all been affected by trypsin.

2. — Phospholipase C action

During a preliminary assay at 37 °C for 60 min, the level of viral inactivation as a function of enzyme concentration reached a plateau at 100 µg/ml. In Fig. 4 are presented inactivation kinetics at 10 and 100 µg/ml. The decrease in infectivity given by 100 µg/ml phospholipase is on the order of 2 log units at 37 °C for 60 min for both the Alfort

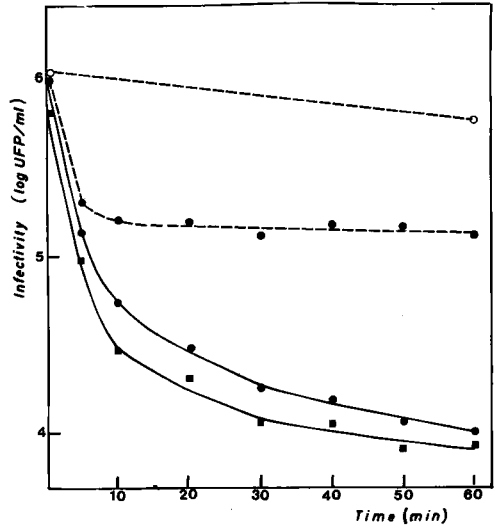


Fig. 4. Inactivation kinetics of HCV at 37 °C in the presence of 10 (---) or 100 (—) µg/ml of phospholipase C. Strains Alfort (●) or Thiverval (■). 0 : Alfort strain + 100 µg/ml of phospholipase C + 1,5 mM EDTA.

and Thiverval strains. Residual infectivity is insensitive to ribonuclease and remains neutralizable by an anti-serum (results not shown). The incomplete inhibition of the reaction by EDTA (seen in Fig. 4), reputed to efficient at the concentration employed, can be at least partially explained by the protease contamination we were able to demonstrate.

Discussion

— *Trypsin*. The inactivation kinetics reported above demonstrate a considerable decrease in infectivity of HC viral suspensions treated with trypsin, the concentration of enzyme being similar to that used in earlier studies on other viruses (Gresser and Enders, 1961 ; Cartwright *et al.*, 1970 ; Maeno *et al.*, 1970 ; Nermut, 1972). This particular sensitivity to proteolytic treatment should be compared to the low resistance of HCV towards putrefaction. This could also partially explain the negative results of oral vaccination (Corthier, 1974).

The utilization of extemporaneously made trypsin solutions in a protein-poor reaction medium has probably contributed to a more accurate assessment of the extent of inactivation. In this respect, our results are comparable to those previously obtained with the HCV (Van Bekkum and Barteling, 1970) and

with the closely-related Mucosal Disease virus (Darbyshire, 1960 ; Dinter, 1963 ; Horzinek *et al.*, 1971 ; Corthier *et al.*, 1974) obtained by Hafez and Liess (1972).

It had generally been admitted that only the Flavivirus group within the *Togaviridae* exhibited a generalized sensitivity of their hemagglutinating and infectivity towards trypsin (Cheng, 1958 ; Hannoun, 1968). Indeed, some Alphaviruses also show this sensitivity (Gorman and Goss, 1972) ; so, this criterion cannot provide significant taxonomic information.

Two slopes of inactivation are revealed by our kinetic studies. Such a step-wise decrease of infectivity resulting from an inactivating treatment is not uncommon in virus field. It can be explained by either a qualitative (non-equivalent sites : Dimmock, 1967 ; Flemming, 1971) or a quantitative (phenotypic mixture) decrease of the infectivity of the suspension. The use of purified viral suspensions is nevertheless implicated in the detailed study of these phenomena.

— *Phospholipase C*. The extent of HCV inactivation after incubation with phospholipase C is similar to that reported for another Togavirus, Equine Arteritis virus (Hyllseth, 1973). The sensitivity of Togaviruses to this enzyme is related to the presence of lecithins and sphingomyelins among membrane phospholipids (Pfefferkorn and Hunter, 1963 ; David, 1971) derived from the host cell (Strauss *et al.*, 1968). The more or less immediate inactivation of infectivity resulting from phospholipase C action in this family is accounted for by the variable degree of cohesion assured by phospholipids at the level of the particle. Thus, the deterioration of the lipid component of the Semliki Forest virus leads to a delayed decrease of infectivity which takes place only when the stability of the viral structure is compromised (Friedman and Pastan, 1969 ; Kennedy, 1974). On the other

hand, hydrolysis of the phospholipids of Murray Valley Encephalitis virus (Flavivirus) appears to precociously affect its infectivity (Anderson and Ada, 1961). Inactivation kinetics of HCV, even at low enzymes doses, suggest that the virulence of the particle requires membrane phospholipid integrity.

— *Comparison among strains*. Our results show that not only is there a marked trypsin sensitivity of the virus, but that it is equal for pathogenic and attenuated strains. In the case of Transmissible Gastroenteritis, however, a different situation has been observed, since resistance to proteolytic action serves as a clear marker of the virulence of strains (Furuuchi *et al.*, 1975). Otherwise, the two HCV strains exhibit an inactivation of equal intensity in the presence of phospholipase C.

The action of these two enzymes, as that of pH changes, is exercised essentially at the level of the superficial structures of the virion. Until now, only heat treatment (56°C-60 min) enabled a distinction to be made between these two HCV strains (Aynaud, 1972) ; with the Foot and Mouth Disease virus, for example, the effect of different pH values has likewise enabled certain heat-sensitive strains to be distinguished from the wild-type (Asso, 1967). The mutation is thus located at the level of the external surface of the virus (Laporte, 1973). This fact suggests, in the case of HCV, that the "56°C fragile" mutation could be expressed at the level of capsid proteins : among all the agents studied, only heat is capable of affecting them directly.

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Summary

The actions of trypsin and phospholipase C on the infectivity of Hog Cholera virus (HCV) were studied. Inactivation kinetics reveal a marked decrease of the infectivity of HC virus in the presence of these two agents. The particular sensitivity of this virus towards proteolytic action sheds light on certain of its behavior characteristics in the field. Virus infectivity seems to be dependent on the integrity of membrane phospholipids. No relation was observed between the rate of inactivation and the pathogenicity of the strains.

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