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Thermal Inactivation Studies of a Coronavirus, Transmissible Gastroenteritis Virus

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SUMMARY

The thermolability of transmissible gastroenteritis virus (TGEV) was studied between 31 and 55 °C using two different strains. The loss of infectivity followed first order kinetics except at the highest temperature. The values of the thermodynamic parameters indicated that the mechanisms involved above and below 45 °C are clearly distinct. The rates of inactivation were greater at alkaline than at neutral pH, yet the nature of the reaction appeared unchanged. Using four independent stocks of mutagenized virus, we failed to select thermal-resistant mutants by survivor selection at 38 °C. In contrast, thermal-resistant mutants were consistently obtained at 54 °C. However, the latter did not show any increased stability at 38 °C, confirming the fact that a different inactivation process takes place at high and at physiological temperatures.

INTRODUCTION

Earlier studies on the effect of heating RNA viruses led to the conclusion that inactivation between 30 and 60 °C generally takes place according to two thermodynamically distinct mechanisms, depending on the temperature range. Infectivity degradation at 'low' temperature is considered to be caused selectively by denaturation of the nucleic acid, whereas functions related to the protein components of the virus would be primarily lost at 'high' temperature (Dimmock, 1967; Ginoza, 1968; Flemming, 1971).

The present report is concerned with thermal inactivation of transmissible gastroenteritis virus of swine, a member of the coronaviridae. This family comprises pathogenic, enveloped RNA viruses having a large single-stranded one-piece genome of positive polarity (for reviews, see Tyrrell *et al.*, 1978; Pensaert & Callebaut, 1978). Thus far, no detailed information is available on the thermal inactivation process of a coronavirus. Our results indicate that (i) a different thermodynamic reaction is involved above and below 45 °C and (ii) thermal-resistant mutants are easily selected in the 'high' temperature range, but not in the 'low' temperature range.

METHODS

Viruses and cells. The Purdue and the D_{52} strains were used as the source of transmissible gastroenteritis virus (TGEV). The virus was propagated in the pig kidney cell line RP_D, and maintained in Eagle's minimum essential medium (MEM) containing 2% calf serum; the cultures were frozen at −70 °C at the first signs of c.p.e. Infectivity was quantified by plaque formation on the pig kidney cell line RP_{TG}, as described elsewhere (Laude *et al.*, 1981).

Thermal inactivation. Supernatant fluids from infected cultures were thawed and then clarified by low-speed centrifugation before dilution 1:10 in a 50 mM-HEPES solution (Sigma) buffered at pH 7 or 8, and supplemented with antibiotics. For short incubations this

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solution was preheated before adding the virus. One ml amounts in glass tubes were placed in well-stirred water baths in the dark. Duplicate aliquots were removed at given times and stored at −70 °C until titration of infectivity. pH variation did not exceed ±0·03 pH unit during the heat treatment.

Treatment of data. When a virus is inactivated according to a first order reaction, the rate constant is given by the formula:

$$k' = -(\ln Vt/Vo)t$$

where Vo is the original concentration of infectious virus and Vt the concentration of surviving virus at a given time t. The rate constant k' and the thermodynamic parameters are respectively related to the activation equilibrium constant $K^{\neq}$ defined by the Eyring's theory, according to the equations:

$$(1) \quad k' = (k\mathrm{T}/\mathrm{h})\, K^{\neq}$$
$$(2) \quad \Delta G^{\neq} = \Delta H^{\neq} - \mathrm{T}.\, \Delta S^{\neq} = -\mathrm{RT}\ln K^{\neq}$$

with $\Delta G^{\neq}$, the Gibb's function change; $\Delta H^{\neq}$, the enthalpy change; $\Delta S^{\neq}$, the entropy change; T, the absolute temperature; and k, h and R, known constants. By combination and development of (1) and (2) (see Ginoza, 1968 for details), the thermodynamic parameters can be deduced from the measure of the rate constant k' at any given temperature:

$$\Delta G^{\neq} = 4{\cdot}58\ \mathrm{T}\ (10{\cdot}32 + \log \mathrm{T} - \log k')$$

$$\Delta H^{\neq} = 4{\cdot}58\ [\mathrm{d} \log k'/\mathrm{d}\ (1/\mathrm{T})] - 1{\cdot}98\ \mathrm{T}$$

Preparation of mutagenized virus stocks. A suspension of concentrated plaque-purified D_{52} virus (2×10^8 p.f.u./ml) was treated with nitrous acid (0·5 M) at pH 4·2 in sodium acetate buffer using a procedure similar to that described by Thiry (1963). A linear infectivity decay of 1 log p.f.u./min at 25 °C was observed, while the virus is known to be stable at pH 4·2 (Laude *et al.*, 1981). The reaction was stopped after 1, 2, 3 and 4 min by diluting the mixture 1 : 10 in MEM. The residual virus was immediately allowed to adsorb on RP_D monolayers (5 ml of each suspension per 2×10^7 cells). The cultures were frozen 48 to 60 h post-infection. The resulting mutagenized virus stocks were called M_1 to M_4.

Selection of thermal-resistant mutants. Each virus stock was independently subjected to several cycles of survivor selection at 54 °C and 38 °C. Heating was performed as described above, except that undiluted virus suspensions were used, the pH of which was carefully adjusted to 7·5 prior to the treatment. After each inactivation step, limited on purpose to 1 to 2 logs, the residual virus was immediately passaged on RP_D cell cultures, which were frozen 48 h post-infection.

RESULTS

Inactivation of infectivity

The rate of inactivation of TGEV was measured in the temperature range of 31 to 55 °C (Fig. 1*a*). An exponential decrease of infectivity, without an initial shoulder, could be observed between 31 and 51 °C. Thus, the inactivation proceeded as a first-order reaction, implying that the virus preparations were homogeneous. At a higher temperature (55 °C) a two component curve was observed (omitted for clarity), a finding which has been occasionally recognized with some other RNA viruses (see Ginoza, 1968). Thermal inactivation of the virus at pH 8 showed that the half-life at any temperature is markedly reduced (Fig. 1*b*). Pretreatment of the samples for 10 min at pH 8 did not influence the inactivation pattern at pH 7 (data not shown).

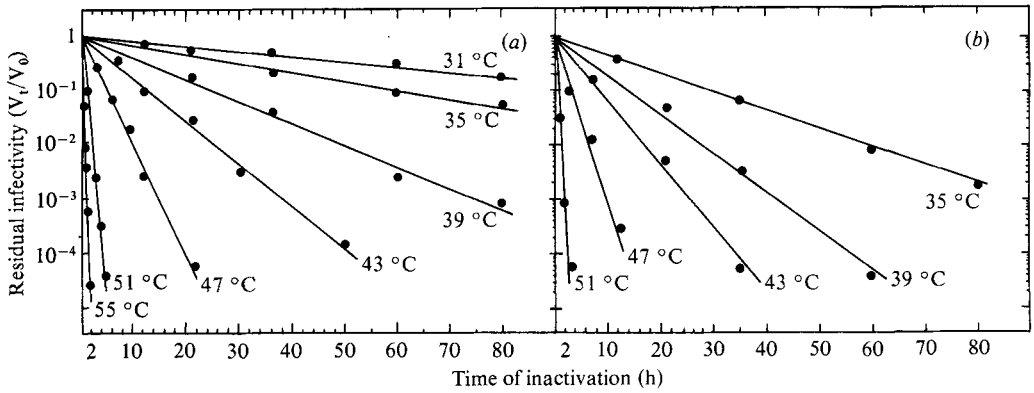

Fig. 1. Temperature dependence of the thermal inactivation rate of TGEV (D_{52} strain) between 55 and 31 °C. Virus diluted in a HEPES buffer solution at (*a*) pH 7 or (*b*) pH 8.

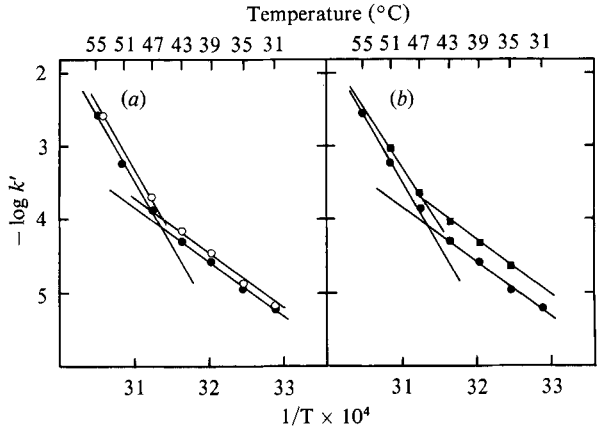

Fig. 2. Arrhenius plot for thermal inactivation of TGEV. Logarithms of rate constants were plotted against reciprocal of absolute temperature. The k' values were derived from graphs such as shown in Fig. 1 (for 55 °C, the k' value corresponds to the fast inactivation component). ●, D_{52} strain heated at pH 7; ■, D_{52} strain heated at pH 8; ○, Purdue strain heated at pH 7.

Table 1. *Thermodynamic parameters for the inactivation of TGE virus (D_{52} strain, pH 7)*

Temperature	$\Delta G^{\neq}$ (kcal/mol)	$\Delta H^{\neq}$ (kcal/mol)	$\Delta S^{\neq}$ (cal. mol/°C)
Low range (38 °C)	26	33	23
High range (54 °C)	23	81	177

The different velocity constants were plotted against the temperature according to an Arrhenius diagram. As shown in Fig. 2, the plot is composed of two straight lines which intercept at about 45 °C. The low cell culture-passaged D_{52} strain and the high cell culture-passaged Purdue strain displayed a similar pattern of inactivation (Fig. 2*a*). The slopes of the curves, which are related to the enthalpy change, are not significantly modified when inactivation is carried out at alkaline pH (Fig. 2*b*). The values determined for the thermodynamic parameters are shown in Table 1.

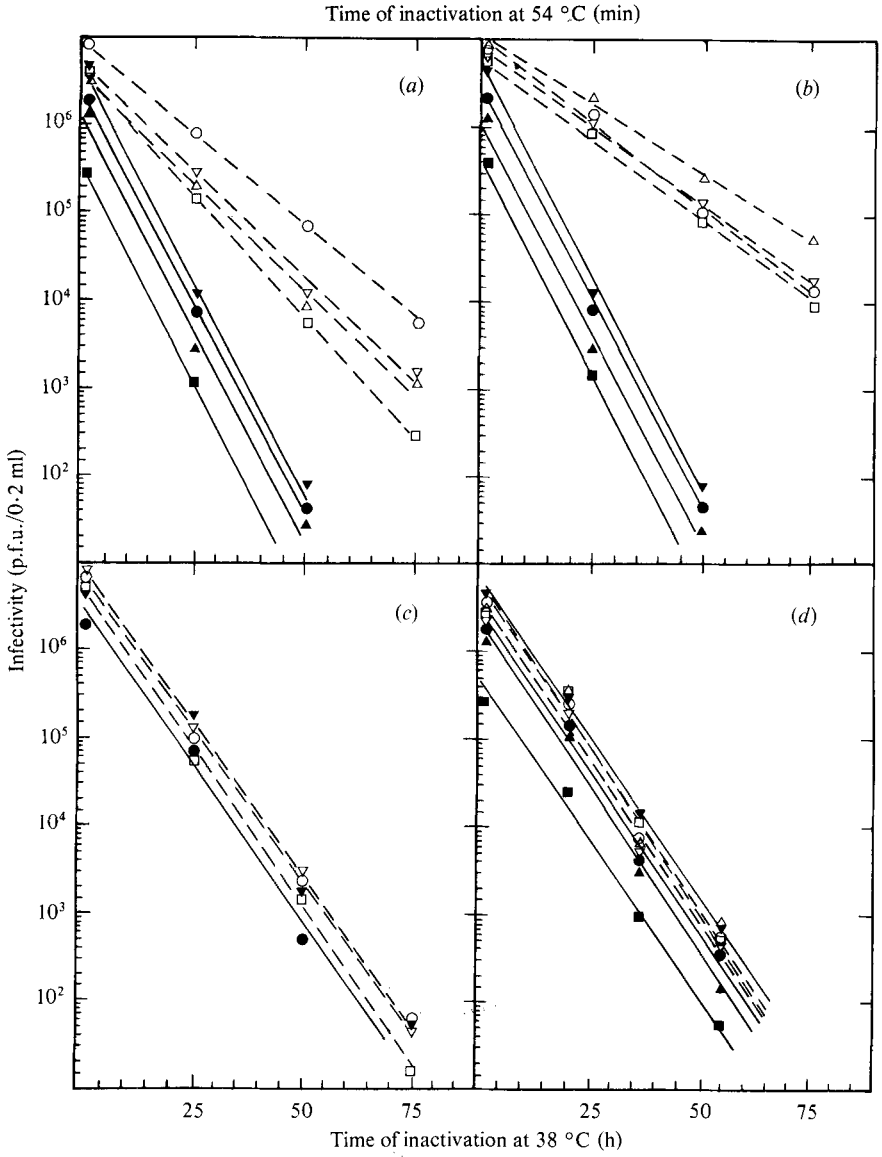

Fig. 3. Effect of serial survivor selection experiments on the thermolability of TGEV at 54 and 38 °C (undiluted virus, medium buffered at pH 7·5). Four independent mutagenized virus stocks were used: M_1 (●), (○), M_2 (▲, △), M_3 (■, □) and M_4 (▼, ▽). Each panel shows the destruction of infectivity of the virus parent lines (——) and of the progeny after survivor selection cycles (– – –). (*a*) 4 cycles at 54 °C; (*b, c*) 8 cycles at 54 °C; (*d*) 8 cycles at 38 °C.

Selection of thermal-resistant (tr) *mutants*

An attempt was made to produce *tr* mutants at 38 and 54 °C, two temperatures chosen as being roughly equidistant from the intersection point of the Arrhenius diagram. As shown in Fig. 3, *tr* mutants at 54 °C were obtained from the 4th cycle of survivor selection (Fig. 3*a*). The mean 50 min survival of the 8th passage material was about 1000-fold higher than that of the parental viruses (Fig. 3*b*). However, none of these mutants showed altered characteristics of the thermal inactivation rate at 38 °C (Fig. 3*c*). In contrast, no increased resistance at

38 °C resulted from survivor selection experiments at this temperature, whichever mutagenized stock was used (Fig. 3*d*), even after 15 cycles (not shown).

DISCUSSION

Thermal inactivation of a coronavirus essentially resembles that observed previously with several other RNA viruses. TGEV thermal inactivation is mediated by two thermodynamically distinct reactions below and above 45 °C. This temperature, at which both reactions proceed at similar rate, was found to be nearly the same for the other RNA viruses studied (between 40 and 45 °C; Bachrach *et al.*, 1957; Dougherty, 1961; Dimmock, 1967; Ginoza, 1968; Flemming, 1971; Walder & Liprandi, 1976).

The mechanisms underlying the loss of infectivity on either side of this temperature have not yet been fully elucidated. The analysis of RNA and of antigenic properties of heated virions which have been carried out with a few virus models supports the idea that inactivation is based upon a random degradation of the nucleic acid at low temperature, whereas a greater change in the conformation of the virus structure would occur at high temperature (Brown & Wild, 1966; Dimmock, 1967; Bader & Steck, 1969; Flemming, 1971; Denoya *et al.*, 1978). Although in this study nothing was attempted in this way, the values determined for the thermodynamic parameters controlling TGEV inactivation by heat clearly show that the target affected below 45 °C is different from that affected above 45 °C (Table 1). Furthermore, the value of enthalpy variation obtained in the low temperature range is compatible with that known for the heat-induced hydrolysis of free RNA ($\Delta h^{\neq} = 30$ kcal/mol; Eigner *et al.*, 1961). Additionally, we showed that, despite the pronounced effect exerted by an alkaline pH on the rate of TGEV thermal inactivation (our results; Pocock & Garwes, 1975), there is no apparent differential mechanism that is associated with this process (Fig. 2*b*).

A more intriguing feature of this study is that, whereas survivor selection procedure from nitrous acid-treated virus pools easily induced *tr* mutants at high temperature, it consistently failed to produce *tr* mutants at 'physiological' temperature. Conceivably, this could be related to the nature of the target which is hit below 45 °C. The fact, as stated above, that RNA degradation could be responsible for the loss of infectivity in the low temperature range, does not supply by itself a sufficient explanation for the apparent impossibility of selecting viable *tr* mutants at 38 °C; there is evidence that thermal stability of virus RNA may not only be a function of its intrinsic properties, but may also depend on its interaction with virus ribonucleoprotein (Dimmock, 1967; Flemming, 1971). An alternative explanation for our observations might be the presence of an endoribonuclease activity located within the virus particles, as postulated by Denoya *et al.* (1978) in the case of foot-and-mouth disease virus.

In any case, the following facts could be drawn from a survey of the literature on that particular point. (i) Whereas numerous *tr* mutants have been isolated at high temperature (42 to 56 °C), there is to our knowledge no example of *tr* mutation at a temperature close to 38 °C. (ii) Several studies using different strains of Venezuelan equine encephalitis virus and TGEV underlined a significantly more uniform inactivation rate at 37 °C, as compared to that observed at higher temperature (Walder & Liprandi, 1976; Laude *et al.*, 1981). (iii) Finally, Youngner (1957) mentioned that he was unable to select a poliovirus *tr* mutant at 38 °C. In conclusion, the question arises whether such a situation could be a general feature among RNA viruses.

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