Inactivation and Reactivation of Semliki Forest Virus by Urea and Guanidine Hydrochloride

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SUMMARY

The inactivation of Semliki Forest virus in solutions of guanidine hydrochloride and urea shows an anomalous dependence on concentration. Under some circumstances the inactivation is reversible. This reactivation is non-cooperative and extracellular and can be induced by incubation at high, or at negligible inactivant concentrations. It is suggested that changes in the surface structure of the virus particle take place before or during inactivation and that modifications of the conformation of the virus are involved in the reversible reactions.

INTRODUCTION

The multiplicity reactivation of several animal viruses has been reviewed by Sharp (1968). The original description of this phenomenon by Luria (1947) concerned reactivation of bacteriophage following inactivation by ultraviolet (u.v.) irradiation; since then, inactivation by u.v. light has been favoured to demonstrate similar effects with animal viruses. Henle & Liu (1951) demonstrated multiplicity reactivation for influenza virus after thermal inactivation at 56°, but Drake (1958) could not detect this effect for poliovirus after heating at 50°. Non-genetic reactivation, in which a virus inactivated by degradation of a protein component has its infectivity restored by the action of non-genetic element of a second virus particle, has been demonstrated for poxviruses (Fenner, 1962; Ronn, Inglot & Lycke, 1970). Both multiplicity and non-genetic reactivation thus rely on the intracellular cooperation of variously degraded virus particles.

By contrast, this paper describes the extracellular reactivation of Semliki Forest virus after inactivation in solutions of guanidine hydrochloride or urea, under conditions that preclude multiplicity reactivation. In these studies inactivation or reactivation is recognized by reduction or recovery of the ability to form plaques.

METHODS

Virus. Semliki Forest virus was that used in previous thermal inactivation studies and designated v14L (Fleming, 1971). The method of assay in agar (Oxoid no. 1) suspensions of chick embryo cells has been fully described (Bradish, Allner & Maber, 1971); the infectivity of the virus stock was $10^8$ p.f.u./ml. The highest concentration of virus used in the plaque assay was 0.1 p.f.u./cell. Concentrations of guanidine hydrochloride (guanidine HCl) or urea in the assay system did not exceed 150 $\mu$g./ml. or 3 mg./ml., respectively; these concentrations did not influence the assay of untreated virus.

Inactivation. Solutions of guanidine HCl and urea were freshly prepared in sterile phosphate buffer solutions (PPB), pH 7.5, final ionic strength 0.1. These were used without further
sterilization. Virus samples were inactivated in thin-walled test tubes in well-stirred water baths by addition of 1 vol. of virus suspension in its original medium (Earle's saline containing yeast extract plus tryptic meat broth with 10% calf serum; EYT, CS), or after dilution in Parker's medium 199 with 10% calf serum (199, CS), to 9 vol. inactivant solution. Inactivation was stopped by a tenfold dilution in 199, CS (Fig. 1, 2).

Zonal sedimentation analysis. Density gradients of 5 to 30% sucrose in PPB with 0.1% bovine plasma albumin (BPA, Armour, Fraction V) were prepared from six 4 ml. portions and allowed to diffuse overnight at 4°. Gradients for isopyknic banding were prepared as above from 35 to 50% sucrose solutions and used without diffusion. These were overlaid with 1 ml. of virus solution immediately before centrifugation at 25,000 rev./min. in a Beckman/Spinco SW 25 rotor at 0°. After velocity sedimentation for 1 hr or isopyknic banding for 16 hr, approximately 1 ml. samples were collected into 9 ml. of 199, CS through a hole in the bottom of the tube.

Neutralization by antiserum. The preparation of antiserum has been described. The ability of antiserum to neutralize virus infectivity was also measured as described (Fleming, 1971) except that virus + antiserum mixtures were incubated at 20° to prevent reactivation.

RESULTS

Effect of concentration of inactivant

The residual infectivity after exposure to various concentrations of guanidine HCl at 28° is shown in Fig. 1. As the time of exposure was increased from 2 to 20 min. the response characteristic changed from a conventional sigmoid curve to a 'saxophonic' curve showing a maximum inactivation at a definite concentration of guanidine HCl.

A similar anomalous response was found for the action of urea during determination of the temperature dependence of the concentration having maximum effect (Fig. 2). Despite difficulties in the standardization of these results, the effect of temperature, in this context, was marginal. The main difference between the effects produced by urea and guanidine HCl was in the level of residual infectivity at high concentration.

Kinetics of inactivation in guanidine HCl and urea

The inactivation of infectivity at 28° in 16% guanidine HCl and 30% urea (concentrations at the maxima, C1, in Fig. 1 and 2) was initially rapid and subsequently very slow. Inactivation by guanidine HCl at the critical concentration (C1) that produced maximum loss of infectivity, the result of increasing the inactivant concentration from C1 to C2 during inactivation, and the effect of re-adjusting to C1 after it had been raised to C2 are shown in Fig. 3. The reversibility of the reaction is clear; equilibrium was reached far more rapidly at C1 than at C2 but was not affected by the time of heating at the alternative concentration. Similar results were obtained in solutions of urea. The anomalies in Fig. 1 and 2 were due to these reversible reactions.

Inactivation at 0° in 16% guanidine HCl produced an equilibrium infectivity approximately seven times higher than that at 28°; it was necessary to prolong exposure beyond 4 hr to cause greater inactivation in 10% than 16% guanidine HCl at this lower temperature.

Reactivation by dilution and heating

After inactivation, infectivity could also be increased at agent concentrations that did not influence the stability of the virus (Fig. 1 and 2). Thus, the infectivity of a virus sample was reduced from 10⁶ to 10⁵ p.f.u./ml. in a 10% solution of guanidine HCl at 28°; the guanidine
HCl concentration was then reduced to 1% by dilution in 199, CS and the solutions heated at the temperatures shown in Fig. 4. Infectivity increased by the same factor as at C₂ (Fig. 3), and the extent of the increase was the same if the solutions were diluted further.

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**Fig. 1. Inactivation of SFV in guanidine HCl solutions.** Virus diluted 1/10 in 199, CS before addition. Infectivity assayed after 2 min. (○); 6 min. (▲); 12 min. (■); and 20 min. (○) at 28°C.

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**Fig. 2. Inactivation of SFV in urea solutions.** Virus diluted 1/10 in 199, CS before addition. Infectivity assayed after 15 min. at 35°C (○); 90 min. at 28°C (▲); and 4 hr at 19°C (■).
before heating. Virus infectivity could thus be increased by a factor of 100 or more after extended exposure in 10% guanidine HCl.

**Reactivation during assay procedure**

The increase of infectivity by heating at 35° was surprising since the assay procedure involved a relatively prolonged incubation at this temperature in a suspension of chick embryo cells in agar. To determine whether the increase could occur in agar, a virus sample inactivated as above was incubated for 2 hr at 35° in 0.6% agar before overlaying with a suspension of cells. In a control experiment with untreated virus the plaque count was depressed to 2.5%; with virus inactivated as above the count was doubled. This 80-fold difference indicates that reactivation occurred in the agar gel but was blocked in normal assays by adsorption to cells before the change could be induced. The low efficiency of elution of untreated virus from agar, and the low plaquing efficiency of SFV, do not justify further interpretation.

**Sedimentation properties following inactivation and reactivation**

The component undergoing inactivation and reactivation was identified by its sedimentation velocity as single virus particles. The reactivated virus, the virus yet to be reactivated (reactivable) and the residual infectivity after inactivation all sedimented with a slightly greater velocity than the untreated virus. Isopyknic banding experiments showed that the density of the reactivated and reactivable virus was 1.188 g./ml. compared with 1.173 g./ml.
for the untreated virus (Fig. 5). This increase was sufficient to cause the higher sedimentation velocity noted above. Since the sedimentation coefficient of the whole virus is 275S, that of virus cores 140S, and of virus RNA 46S (Friedman & Berezesky, 1967), the possibility of significant breakdown of virus in these experiments was discounted. Infective SFV-RNA also

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**Fig. 4.** Thermal reactivation of SFV. The infectivity was reduced from $10^8$ to $10^5$ p.f.u./ml in 10% guanidine HCl at 28°; after 1/10 dilution in 199, CS, the solutions were heated at the temperatures shown.

**Fig. 5.** Isopyknic banding patterns of SFV after various treatments. Control, untreated virus (■); virus after inactivation from $10^8$ to $10^5$ p.f.u./ml in 10% guanidine HCl at 28°, dilution in 199, CS and reactivation at 35° (○); virus after inactivation as above and reactivation of the eluted fractions at 35° after a tenfold dilution in 199, CS ($\triangle$).
requires hypertonic conditions for adsorption to cells and neither RNA nor virus cores would form plaques under the assay conditions used.

It is significant that virus could be reactivated after sedimentation or partial purification by electrophoresis or chromatography so that it was unlikely that a second component was involved in this reaction.

**Surface properties following inactivation and reactivation**

As exposure to 10 % guanidine HCl at 28° was prolonged, the virus remaining infective became less readily neutralized by rabbit antiserum at 0.02 µl./ml. (Fig. 6). The rise in the fraction of residual infectivity was not due to blocking of antibody by non-infective virus particles since untreated virus added to the system was neutralized as by antiserum at 0.02 µl./ml. Virus inactivated from 10⁸ to 10⁵ p.f.u./ml. in 10 % guanidine HCl at 28°, and reactivated by dilution and heating, was also less readily neutralized, but although the differences of sensitivity to antiserum between original and treated virus were significant at all antiserum dilutions up to 0.4 µl./ml., the responses of inactivated and reactivated virus could not be differentiated.

![Figure 6](image)

*Fig. 6. Neutralization of SFV by rabbit antiserum after exposure to guanidine HCl. Virus was heated in 10 % guanidine HCl at 28° to give prior inactivation defined by log V₀/Vₜ (△). Untreated virus was added to some solutions before neutralization (○).*

Virus reactivated after exposure to 10 % guanidine HCl was more sensitive to heating at 46° than the untreated virus. Although the properties of SFV in thermal inactivation cannot always be determined unequivocally after treatment in guanidine HCl, this increased lability is a further indication of a modification of the surface structure of the virus (Fleming, 1971).

**Effect of polyions on plaque assay**

SFV, inactivated and reactivated by dilution and heating as previously described, was diluted in 199, CS to give approximately 50 p.f.u./ml. when assayed using 0.75 % agar-Noble in medium 199 containing 5 % calf serum (Fleming, in preparation). The influence of sodium dextran sulphate 2000 and DEAE-dextran on the original, inactivated and reactivated virus is seen in Fig. 7. All three viruses showed a qualitatively similar response in sodium dextran sulphate, but addition of DEAE-dextran caused changes of plaque count that depended on virus treatment. Virus that still remained infective after exposure to guanidine HCl suffered a surface change that altered the response from one of a gradual decrease of plaque count as
the DEAE-dextran concentration was raised, to one with a maximum count at 120 μg./ml. During reactivation changes occurred that caused this response to return towards that of the untreated virus.

Sensitivity to guanidine HCl after passage in chick embryo cells

Passage of SFV (v 14.1) in chick embryo cells produced virus more resistant to inactivation by 16 % guanidine HCl at 28°. Thus, under these conditions, resistance to guanidine HCl inactivation is partly a phenotypic expression of cellular origin, and this may be exploited to produce virus populations susceptible to surface modification by guanidine HCl with minimum loss of infectivity.

![Graph](image_url)

**Fig. 7.** Assay of SFV in the presence of polyions after various treatments. Control, untreated virus (●); virus after inactivation from 10⁸ to 10⁵ p.f.u./ml. in 10% guanidine HCl at 28° (△); virus after inactivation as above, dilution in 199, CS and heating at 35° to induce maximum reactivation (▲).

**DISCUSSION**

Retention of infectivity alone cannot be accepted as evidence that structural changes have not occurred in virus particles; for instance, hydrolysis of phospholipids may not destroy the infectivity of SFV (Friedman & Pastan, 1969). Similarly, the complete recovery of infectivity after exposure to low concentrations of guanidine HCl and urea, and the residual infectivity during inactivation, may relate to virus particles of modified structure; this is demonstrated by the increase in the density of guanidine HCl treated, and 'repaired' infective particles.

The sensitivity of viruses to inactivating agents, such as urea and guanidine HCl, has been correlated with other physical markers and biological properties for poliovirus (Cooper, 1963) and tick-borne encephalitis virus (Mayer & Sabo, 1966). The simplicity of such experi-
ments is attractive, but the determination of the sensitivity is made difficult by the possibility of changes of infectivity at stages after transfer from the inactivating medium. The probability, for instance, of thermal reactivation taking place in the aqueous phase before adsorption, had the plaque assay been carried out on monolayers rather than in cell suspension, has been demonstrated in this paper. This anomalous behaviour shows that more than one property of the virus is involved and that these determine the apparent rate of inactivation and the degree to which reactivation can be induced. The effect of urea and guanidine HCl in the inactivation process is probably to cause a modification of the surface structure which, in turn, increases the thermal sensitivity of the virus.

The changes taking place during reactivation are less easy to identify. The effect of adding DEAE-dextran to agar suspensions of chick embryo cells is to neutralize inhibitors and thereby to broaden the spectrum of cells susceptible to infection (P. Fleming, in preparation); the variation of this effect on reactivation therefore implies a tendency to revert to the initial, more specific, pattern of infection. This may be connected with the observation that some inactivated enzymes can also be reactivated by heating (Pullman et al. 1960). The reversible changes associated with recovery of infectivity are probably related to an early stage of virus replication such as penetration of the cell, or release or unfolding of RNA. Although it is possible that this is a general effect, at least for arboviruses, the necessary combination of appropriate conditions of temperature and inactivant concentration may not be accessible for all systems.

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REFERENCES


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