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Thermoinactivation of Viruses, Communication IV: Factors Determining the Dynamics and Rate of Inactivation of Venezuelan Equine Encephalomyelitis Virus (VEE)

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The effect of a number of biological, chemical and physical factors on the course of the process of infectivity inactivation at different temperatures was studied on the model of VEE virus. Virus populations obtained in HeLa and MASHA cells were found to be less stable to heating at 50 degrees C than those obtained in cells of trypsinized chick embryos and RES cells. Ultracentrifugation resulted in reduced thermostability of VEE virus and increased its sensitivity of thermostabilizing effect of 12.5% magnesium sulphate. Decreasing of pH of the virus-containing suspension from 9.0 to 7.0 slightly slowed down thermoinactivation of VEE virus by the "nucleic" type; more crude changes in pH markedly enhanced inactivation of the virus. In all cases, a change in the duration and dynamics of the process of thermoinactivation of VEE virus occurred owing to a regular change of a limited number of mechanisms of the loss of infectious activity, the specific manifestations of which are discussed.

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The complicated structure of virus particles represents a nucleoproteid complex, in a series of cases surrounded by an outer lipoproteid casing, and it finds a reflection in the variety of machanisms causing a loss of infectious activity of the virions at different temperatures and in different conditions of incubation. As was indicated earlier (2 - 4, 6 - 8), the virus particles become inactive either as a result of previous breakdown of the viral nucleinic acid, (the nucleinic type of inactivation) or as a result of the denaturing of virus proteins (protein type of inactivation). A significant influence on the character and the speed of inactivation is the outer formation of the encased viruses (11).

Varying biological, physics and chemical factors, acting on the process of heat loss of infectious activity of viruses, realize their influence on the basis of one or another type of inactivation, subordinate to sufficiently determined regularities. In this report we present the results of research on the influence of a series of factors on the level of thermostability of the VEE virus. Choice of the model was determined by the circumstance that at a temperature of 50 to 56 degrees, the dynamics of inactivation of the VEE virus allows simultaneous determination of the action of the factors under study on various inactivation machanisms and various structural formations of the virus particles (2, 9).

Materials and Methods

The VEE virus was received from the collection of the Rockefeller Institute in 1944. Taking into account the heterogeneity of the parent virus population (3), from it was made a variant t^s (3, 4) by means of three-fold passivation from platelet to platelet. The virus which was

taken from one platelet at the third passage and replicated in trypsinized chicken embryo cells (TKE) was used as the mother virus. In all cases where there was no special indication, the virus was used in the form of a culture liquid taken 24 hours after infection of the TKE cells and incubated at 36 degrees (infection multiplicity 0,0005 PFU/cell). In several experiments we used thermoresistant (t^r) variant of the VEE virus, the receipt and properties of which are described earlier (4).

Preparation of the TKE cells, the heating, the cultivation and the titration of the VEE virus were done according to the methods described earlier (2, 4).

Cells of the intertwining lines of RES, HeLa, and MASHA were grown in matras flasks at a volume of 100 ml. under a layer of feeding medium (medium No. 199 with 10% bovine serum). Infection of the cells with the mother virus took place with an infection multiplicity of 5 PFU/cell; incubation of the infected culture a t 36 degrees lasted 48 hours; as an aggregate medium, medium No. 199 with 2% bovine serum was used.

To establish the indisepnsable value for pH, we added to the virus-containing liquid a corresponding quantity of 10% solution of NaOH or 1 n of HCl with the subsequent pH control with the aid of a potentiometer.

Cleaning the VEE virus. The virus-containing liquid received during replication of the VEE virus in TKE cells (aggregation medium - medium No. 199 without bovine serum), was centrifuged for 20 minutes at 2000 revs/min. to remove the cell detritus. The remaining liquid was centrifuged for 2 1/2 hours in a carbon rotor 8 x 50 of centrifuge Superspeed-50 at 27,000 revs/min. The precipitate was resuspended in 15 ml. of medium No. 199 and homogenized with the aid of a homogenizer (Downs, 15 oscillations). The suspension was cleared for 15 minutes at 10,000 revs/min. From the remaining liquid, the virus was precipated for 2 1/2 hours at 2 5,000 revs/min using the centrifuge Superspeed-50 (bucket-rotor 3x20) through 16% potassium tartrate on a cushion of 40% potassium tartrate. The virus was gathered from the interphase, homogenized in medium no. 199, clarified as indicated above, and precipitated with the aid of still one more cycle of ultracentrifuging. The precipitate was resuspended in the corresponding medium.

Results

Influence of the cell system on the thermostability of the VEE virus. The virus was replicated in the intertwining RES, HeLa and MASHA cells and in TKE cells. The inactivation dynamics were determined at 50 to 54 degrees. The results of the experiments are presented in sketch 1. The VEE virus (variant t^s) received in various cell models, became inactive at 54 degrees in medium No. 199 with 2% bovine serum with almost identical speed (sketch 1,a). At the same time, the VEE virus (variant t^r) received in TKE cells (in sketch 1,a, the inactivation dynamics are indicated by the broken line), lost its infectious activity significantly more slowly.

At 50 degrees the VEE virus (variant t^r) received in HeLa cells (broken line in sketch 1b), and likewise VEE virus (variant t^s) received in TKE and RES cells, became inactive with the same speed which was noted below as the speed of inactivation of the VEE virus (variant t^s) received in HeLa and MASHA cells.

In this way the speed of inactivation of the VEE virus replicated in various cell systems, was determined by the level of the temperature during heating and by the strain peculiarities of the virus.

The influence of differential centrifuging on the thermostability of the VEE virus. The cleaned virus was resuspended in medium No. 199, diluted 5-fold in a physiological solution and maintained at various temperatures, from 45 to 50 degrees, determining the dynamic of thermo-inactivation. In all cases the speed of inactivation of the cleaned virus was increased by comparison with the control. In sketch 2, the results are presented of the typical experiment on determination of the inactivation of the cleaned virus and the control sample at 50 degrees.

Sketch 3 demonstrates the inactivation of the cleaned VEE virus in various salt solutions at 50 degrees. Addition of 12,5% magnesium sulfate slowed manyfold the loss of the infectiousness of the virions.

Resistance of the cleaned virus in various storage conditions. The cleaned VEE virus was resuspended in Earle's solution with a borax buffer pH 9,0 (1:1) and maintained at a temperature of 37, of 4, and of minus 40 degrees, thawed only once, when at a given period we determined the infectious activity; another part underwent daily thawing and freezing which significantly hastened the dying of the virus (sketch 4, curve 3). The virus at 37 degrees became almost wholly inactive by the following 24 hour period.

At 4 degrees, the virus was more stable that at -40 degrees if the storage period did not exceed 1 - 1 1/2 weeks.

The influence of pH of virus-containing suspensions on the thermo-stability of the VEE virus. In sketch 5a, the graph represents the in-activation of the VEE virus at 53 degrees and pH 7,0, 8,0, and 9,0 during the first 20 minutes of heating. The increase of the pH value leads to the constant growth of the inactivation speed of the virus in these con-ditions.

Sketch 5b demonstrates the virus inactivation at 53 degrees and pH values of 8,0, 10,0 and 6,0 (curves 1, 2, 3 correspond). Obviously, such a large range of pH to the alkaline and also to the acidic side significantly speeds the loss of infectious activity. The virus perishes most quickly in a sharply acidic medium. It should be noted that the angle of curve I (nucleinic type) is less than the mildly sloping part of curve 2, and that the angle of the steep part of curve two (proteid type) in turn is less than the angle of curve 3.

The influence of glutamin and several other additions to virus-containing suspensions on the thermostability of VEE virus. In sketch 6 are represented the dynamics of virus inactivation at 50, 54 and 58 degrees in medium No. 199 with 2% bovine serum in the presence of 0,1% of glutamin. Control statistics are indicated by the broken line. At 54 and 58 degrees, the 0,1% glutamin somewhat speeded the virus inactivation. At 50 degrees before the period of accelerated inactivation of the VEE virus which began after 60 minutes of heating, it appeared that there was a period of

stabilization of infectious titers. However the difference by comparison with the control was small and during use of the titration method of pla- lets, it cannot be recognized as valid.

In connection with the fact that we received similar results 37 degrees, and also for the determination of the influence on vi[illegible]ity of the simultaneous presence of glutamin and magnesium sulfate which exe[illegible]ed a stabilizing action on the protein type of inactivation, we set up a special series of experiments.

The VEE virus grew in TKE cells in Earle's solution with 0,22 % of sodium bicarbonate without serum in order to avoid the influence of the many components usually used in the composition of the growth (aggregate) medium. To the virus-containing suspension we added 0,1% of glutamin, 12,5% magnesium sulfate, 0,1% of bovine a lbumin and their combinations. The virus was heated at 50 degrees for 20 minutes. The results of the experiment are shown in the table. The crystalline bovine albumin had the greatest stabilizing action; this influence was somewhat decreased by the addition of magnesium sulfate. The magnesium sulfate somewhat stabilized the virus;to the same degree, the glutamin speeded its inactivation. The simultaneous introduction of 12,5% magnesium sulfate and 0,1% glutamin led to a notable increase in inactivation.

Discussion

Study of the duration and the thermodynamic parameters of the process of thermoinactivation of viruses leads to the conviction that the loss of infectious activity of the virions as a result of the action of one or another temperature takes place in a limited number of paths or methods which correspond to two basic machanisms of inactivation - the nucleinic, or the protein which reflects the inward structure of the virus particles. Various outward factors exert varying and regular action on the probability of the appearance and interconnection of indicated mechanisms of inactivation (6, 7).

Research on the inactivity curves of infectious activity permits a certain degree of judgement about the nature and mechanisms of th[illegible]s process. Determination of the dynamics of VEE virus inactivation during different temperatures and conditions leads to the conclusion that the change in the angle of one inactivation curve in comparison with another (for instance curves 1, 2 and 3 in sketch 3) within known limits speaks for the acceleration or slowing of the process during the preservation of the mechanism or type of inactivation. From the other hand, sharp change in the angle of one curve in comparison with another is determined at one or another temperature (curves 1 and 3 in sketch 2), and likewise the presence of a break in the curve (for instance, curve 2 in sketch 2 or curve 2 in sketch 4), as a rule, indicates a change in the mechanism itself of the process of thermoinactivation (2 - 4).

In the process of inactivation, the existing action indicates, besides structural components of nucleocapsides of virus particles, their surface lipoproteid complex. The role of surface lipoproteid complex, formed during immediate participation of the cell components (1), appears in various relation to heating at 50 degrees of the virus population received in cells of various origin. For virus populations (t^s [illegible]riant) received in HeLa and MASHA cells, the speed of inactivation taking place,

apparently, according to the protein type, is increased in comparison with viruses from RES and TKE cells which become inactive in these conditions according to the nucleinic type.

On the other hand the difference in thermoresistance of the variants t^r and t^s received in TKE cells is preserved during a transfer to HeLa cells as a system producing viruses. But the temperature level during which the change takes place from one type of inactivation (nucleinic) to another (protein) (2, 3) varies depending on the nature of the cells.

In this way at least in its application to the model under study, the increase of the resistance of virus particles can originate for calculation as an increase in the durability of the protein structure of nucleocapsides (3, 4) as well as an increase in the stability of the surface lipoproteid complex playing the defence role.

The properties of the surface lipoproteid complex determine the formation and duration of the period of induction - of the beginning period of the slowing of the loss of infectious activity of the VEE virus during which it becomes inactive according to the nuclein type. Ultracentrifuging the virus substantially lowers its thermoresistance, at which the period of induction determined for the cleaned virus in the control experiment at 50 degrees disappears and the virus immediately becomes inactive according to the protein type. At the same time the sensitivity of the VEE virus grows - in ordinary conditions it is unclearly expressed (2) - (sensitivity) to thermostabilizing action of magnesium sulfate which lowers the speed of protein inactivation.

Taking into account that in the process of ultracentrifuging during the cleansing of the VEE virus one can observe its "weighting", the change in buoyant density of the virus particles originating apparently because of the chipping out of lipoid components (5, 10), it is possible to suppose that the virus becomes more thermosensitive after the breakdown of its surface lipoproteid complex.

This circumstance complicates work with cleansed virus preparations, inasmuch as the wholeness of the surface lipoproteid complex has great significance during storage of the virus at low temperatures, especially lower than zero degrees (11). If in the course of experimental work it is necessary to remove the suspension of cleansed viruses many times from the refrigerator or if the duration of the work with the suspension does not exceed 1 - 1 1/2 weeks, on the basis of statistics received by us it is possible to recommend a preferred storage at 4 degrees rather than at a temperature lower than zero.

With the model of the foot and mouth virus it was shown that the shift of pH exerts an opposite action on protein and nucleinic types of inactivation (6). In a more acidic medium, the protein inactivation accelerated, and the nucleinic - slowed. In more alkaline mediums, this dependence was opposite. As a result of study done by us on the VEE virus, it was discovered that the changes in pH within known limits did not change the duration of the period of induction and, logically, did not influence the stability of the surface lipoproteid complex. In these conditions the fall of pH lowers the speed of inactivation of the VEE virus according to the nucleinic type. A larger shift of pH (to 6,0 or 10,0) caused destruction of the surface of the lipoproteid complex and change of the mechanism of virus inactivation.

Among the number of tested chemical additives to the virus-containing suspension, the greatest stabilizing action was indicated by crystalline bovine albumin, which slowed the loss of infectious activity of the virus both according to the nucleinic and the protein type. A sufficiently good effect was exerted by the application of a complex of components maintained in medium No. 199.

In our opinion, of particular interest is the search for chemical substances exerting selective action on various types of virus inactivation. If for the protein type, magnesium sulfate has such a selective action, then specificity of the a ction of glutamin on the nucleinic type of inactivation cannot be proved valid. Indirectly, this circumstance can confirm the fact of the neutralization of the stabilizing action of magnesium sulfate by the addition of 0,1% glutamin. It should be taken into account that chemical additives exert action first of all on the surface formations of virions; an increase in the resistance of the surface lipoproteid complex does not change the speed of inactivation of the nucleinic type, but only increases the time during which this inactivation can be determined. The influence on the nucleinic loss of infectiousness is possible only during conditions of sufficient penetrability of the surface structures for the test substance. But this heightening of penetrability can bread down the stability of the surface lipoproteid complex and cause its bremature destruction. Similar interrelations can be traced during the inactivation of VEE virus at 50 degrees in the presence of glutamin (see sketch 6). The induction period is significantly shortened, but during it there is for practical purposes, no defined inactivation of the nucleinic type.

Summing up, it is advisable to note a series of general positions. The resistance of the VEE virus during exposure to low temperatures is determined by the speed of its inactivation of the nucleinic type, at sufficiently high temperatures; when the loss of infectiousness from the first minutes of heating is caused by denaturization of virus proteins, the thermostability is determined in the VEE virus by the speed of inactivation of the protein type. In the interim between these two extreme cases, the resistance of the virus depends in the last analysis on the duration of the induction period and then on that temperature level when the induction period ceases to be determined.

The studied biological, physiological and chemical factors influencing the process of thermoinactivation of the VEE virus speeded or slowed in various conditions, the nucleinic or protein type of inactivation of the virus. On the other hand, their action was realized through the surface lipoproteid complex and was involved in the the change of duration of the induction period, and likewise of the temperature level during which the virus inactivation began to produce principally according to the protein type.

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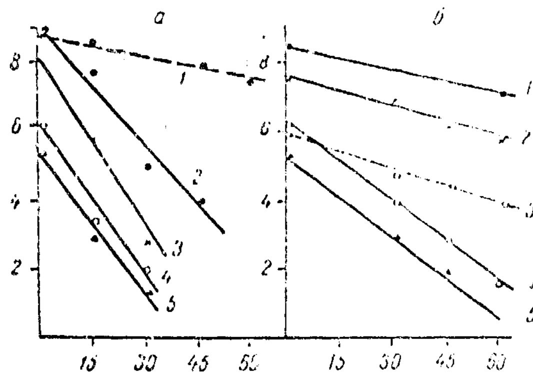
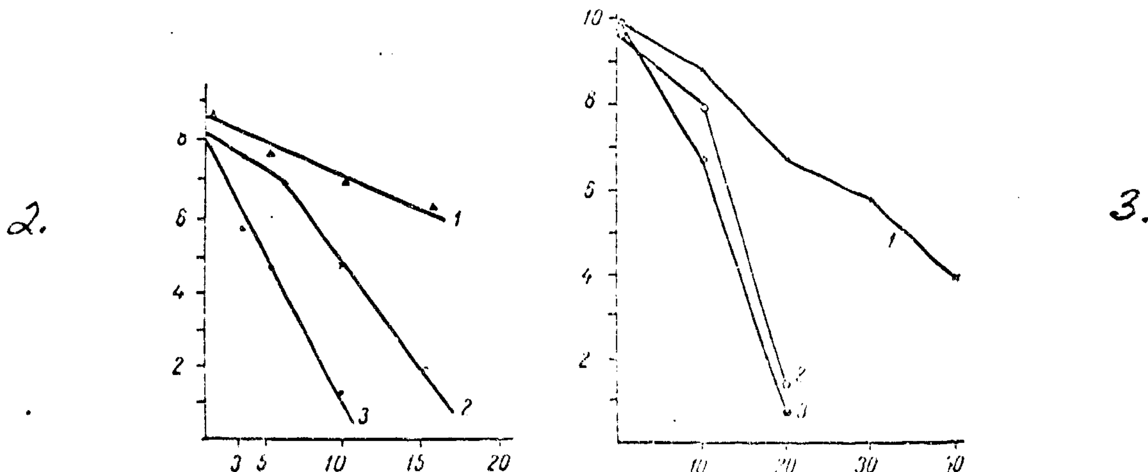
Sketch 1. Inactivation of the VEE virus population, received in cells of various origin.
The broken lineindicates inactivation of the t^r population, the unbroken line - t^s population: a - 54 degrees, the virus received in TKE cells (1 and 2), RES (3), HeLa (4), MASHA (5); b - 50 degrees, virus received in TKE cells (1), RES (2), HeLa (3 and 4), MASHA (5).
Ordinate - virus activity at lg PFU/ml; abscissa - time (in minutes).



Sketch 2. Inactivation of the purified VEE virus at 50 degrees.
1 - parent virus in No. 199 medium without serum; 2 - already diluted in a 5-fold physiological solution; 3 - purified virus in No. 199 medium diluted 5-fold by physiological solution.
Ordinate - virus activity in lg PFU/ml; Abscissa - time (in minutes).



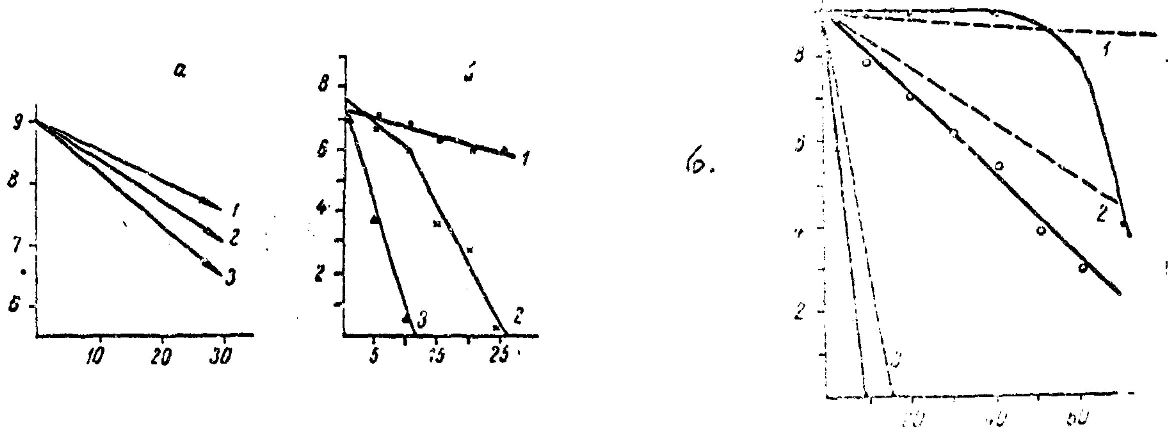
Sketch 3. Inactivation of the purified virus in various salt solutions.
1 - borax buffer, pH 8,8 with 12,5% magnesium sulfate; 2 - borax buffer, pH 8,8; 3 - physiological solution.
Ordinate - virus activity in lg PFU/ml; abscissa - heating time (in minutes).

Sketch 4. Inactivation of the purified VEE virus [illegible] storage.
1 - at 4 degrees; 2 - at -40 degrees; 3 - [illegible] degrees
with daily thawing and freezing; 4 - at 37 [illegible].
Ordinate - virus activity in lg [illegible]; [illegible] - incubation
time (in 24 hour periods)

4.

Sketch 5. Inactivation of the VEE virus with different values of pH at
52 degrees. a: 1 - pH 9,0; 2 - pH 8,0; 3 - pH 7,0; b: 1 -
pH 8,0; 2 - pH 10,0; 3 - pH 6,0.
Ordinate - virus activity in lg PFU/ml; Abscissa - time (in
minutes)

5.



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Sketch 6. Inactivation of the VEE virus at various temperatures in a
growth medium containing 0,1% glutamin.
1 - 50 degrees; 2 - 54 degrees; 3 - 56 degrees. Control
results are indicated by the broken line.
Ordinate - virus activity in lg PFU/ml; Abscissa - time (in
minutes).

Inactivation of the VEE virus at 50 degrees in mediums of various compositions (data are the average results of 3 parallel experiments)

Medium composition in which virus particles are suspended	Fall in infection (in lg PFU/ml) in 20 minutes of heating at 50 degrees
Earle's solution with 0,22% sodium bicarbonate	2,8
The same and 0,1% of bovine albumin	1,8
The same and 0,1% of glutamin	3,1
The same and 12,5% of magnesium sulfate	2,5
The same and 0,1% of Bovine albumin and 12,5% magnesium sulfate	2,3
The same, 0,1% glutamin and 12,5% magnesium sulfate	4,6