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# THERMAL INACTIVATION OF VIRUSES. REPORT 4
# FACTORS ESTABLISHING THE DYNAMICS AND SPEED OF THE PROCESS OF INACTIVATION OF VENEZUELAN EQUINE ENCEPHOMYELITIS (VEE)

by

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# THERMAL INACTIVATION OF VIRUSES. REPORT 4.
# FACTORS ESTABLISHING THE DYNAMICS AND SPEED OF THE PROCESS OF INACTIVATION OF VENEZUELAN EQUINE ENCEPHALOMYELITIS (VEE)

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Admitted June 13 1970

In the model of the VEE virus there is studied the effect of a series of biological, physical and chemical factors on the course of the process of loss of infectious activity with various temperatures. The viral populations, obtained on HeLa and HACHA cells, were shown with less stability to heating in comparison with the viral populations, obtained on cells of trypsinized chicken embryos and RMS. Ultra-centrifuging lead to a reduction of thermal stability of the VEE virus and increased its sensitivity to the thermo-stabilizing action of 12.5 of magnesium sulphate. A decrease of pH of the virus containing suspension from 9.0 to 7.0 somewhat slowed down the thermal inactivation of the VEE virus of the " nuclein" type, more coarse shifts of pH strongly accelerated the death of the virus. In all cases the variations of duration and of the dynamics of the process of the loss of infectious activity of the VEE virus took place as a result of regular variation of a limited number of mechanisms of the loss of infectious activity, the specific effects of which are discussed.

The complex structure of virus particles, representing a nucleo-protein complex, in a series of cases surrounded by an external lipoprotein sheath, find reflection in the difference of the mechanisms causing loss of infectious activity of the virions with various temperatures and conditions of incubation. As determined earlier (References 2 - 4, 6 - 8 ), the virus particles are inactivated either as a result of a primary disturbance of the viral nuclein acid ( " nuclein " type of inactivation ), or owing to the denaturation of the viral proteins ( " protein " type of inactivation ). Considerable effect on the character and speed of inactivation will be shown by the surface formations of the shells of the viruses (Reference 11).

Various biological, physical and chemical factors, acting on the course of the process of heat loss of the infectious activity of the viruses, realize their effect on the basis of that or the other type of inactivation, satisfying sufficiently to definite rules. In the offered report are presented the results of an investigation of the effect of a series of factors on the level of the thermal stability of the VZE virus. The choice of a model depends, by that circumstance, that in the presence of a temperature of 50 - 56°, the dynamics of inactivation of the VZE virus allows simultaneously the establishment of the effect of the studied factors on the different mechanisms of inactivation and of the different structures of formation of the viral particles (References 2,9).

## Material and Methods.

VZE virus obtained from the collection of the Rockefeller Institute in 1944. Taking into account the non-uniformity of the original viral population (Reference 3), from it by 3-fold passage from a patch into a patch was separated the variant $t^s$ (References 3,4). Separated from one patch by a 3-fold passage and bred on Trypsinized Chicken Embryo cells (TCE), we utilized the virus in uterine quality. In all cases, where special conditions are absent, we employed the virus in the form of a cultural fluid, removed within 24 hours after infection of TCE cells, incubated at 36°, (multiplicity of infection 0.0005 EOS / cell). In some experiments we employed the thermo-stable ($t^t$) variant of the VZE virus, the preparat- and properties of which were described earlier (Reference 4).

The preparation of TCE cells, the cultivation and titration of the VZE virus we performed according to the method described earlier (References 2,4).

Cells of the intertwined lines RHE, HeLa, HASHA were bred in matrasses by volume 100 ml under a layer of feed medium( medium - 199 with 10% of bovine serum). The infection of cells by uterine virus we produced with a multiplicity of infection 5 EOS / cell, incubation of the infected cultures at 36° continued 48 hours, in the capacity of the medium of storage we employed medium - 199 with 2% bovine serum.

For the establishment of the necessary values of pH to virus containing fluid we supplemented the appropriate quantities of 10% solution NaOH or 1 normal solution HCl with subsequent control of pH with the aid of a potentiometer.

Purification of VZE virus. The virus containing fluid, obtained by breeding of the VZE virus on TCE cells (medium of storage - medium - 199 without bovine serum), was centrifuged for 20 mins. at 2000 rpm for the elimination of cultural detritus. Supraprecipiated fluid we centrifuged during 2½ hours in the annular rotor 8 x 50 of a centrifuge, Superspeed - 50 at 27000 rpm. The residue we resuspended in 15 ml of medium - 199 and we homogenized by means of a Dauns homogenator (15 oscillations).

The suspended matter we clarified during 15 mins. at 10000 rpm. From supra-precipitated fluid we separated the virus for 2½ hours at 25000 rpm on the Superspeed-50 centrifuge ( rotor tank 3 x 20) through 16% potassium tartrate on a cushion of 40% potassium tartrate. The virus we collected from the interphase, we homogenized it in medium # 199, clarified it, as indicated above, and we precipitated it with the aid again of one cycle of ultracentrifuging. The residue of the virus we resuspended in the appropriate medium.

## Results

The action of the cellular systems on the thermal stability of the VEE virus.

We bred the virus in intertwined RES, HeLa and HASHA cells and on TCE. We established the dynamics of inactivation at 50 and 54°. The results of the experiments are presented in Figure 1. The VEE virus (variant $t^S$), obtained from various cellular models, was inactivated at 54° in medium # 199 with 2% bovine serum with approximately equal speed (Figure 1a). At the same time, the VEE virus (variant $t^F$) obtained on TCE (on Figure 1a the dynamics of its inactivation are indicated by the dashed line) lost its infectious activity considerably more slowly.

At 50° the VEE virus (variant $t^F$ ), obtained on HeLa cells ( dashed line on Figure 1b), and also the VEE virus ( variant $t^S$ ), obtained on TCE and RES cells, were inactivated with one and the same speed, which was noticeably lower from the velocity of inactivation of the VEE virus (variant $t^S$), obtained on HeLa and HASHA cells.

Thus the speed of inactivation of the VEE virus, bred on various cultural systems, is established by the temperature level of the heating and by the strain properties of the virus.

Effect of differential centrifuging on the thermal stability of the VEE virus.

The purified virus we resuspended in medium # 199, we diluted it 5 times with physiological solution and we aged it at various temperatures - from 45 to 50°, determining the dynamics of the thermal inactivation. In all cases the speed of inactivation of the purified virus is increased in comparison with the control. On Figure 2 we presented the results of a typical experiment for determination of the inactivation of the purified virus and of the controlled sample at 50°.

Figure 3 demonstrates the inactivation of purified VEE virus in several salt solutions at 50°. The addition of 12.5% of magnesium sulphate repeatedly retarded the loss of infectiousness of the viruses.

Stability of the purified virus with various levels of storage.

Purified VEE virus we resuspended in Erla solution with a borax



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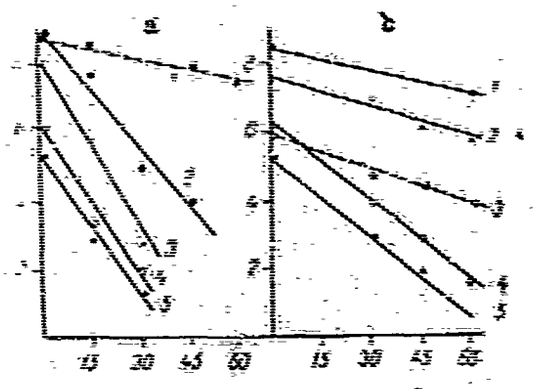
Figure 1 Inactivation of the population of VEE virus, obtained on the cells of various origins.




By the dashed line is designated the inactivation of the $t^T$ population, by the solid lines - the $t^S$ population.

a) - 54°, virus obtained on RDS cells (1 and 2), RDS cells (3), Hela cells (4), HASHA cells (5).

b) - 50°, virus obtained on RDS cells (1), RDS cells (2), Hela cells (3 and 4), HASHA cells (5).

Ordinate axis - activity of the virus in lg ICE /ml, abscissa - time(in mins.)

Figure 2

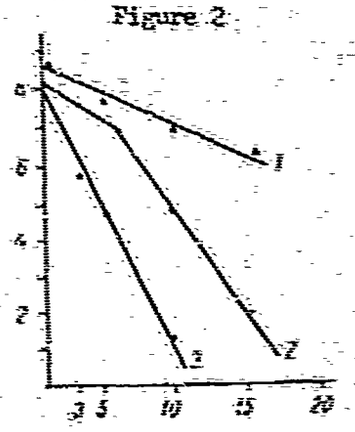
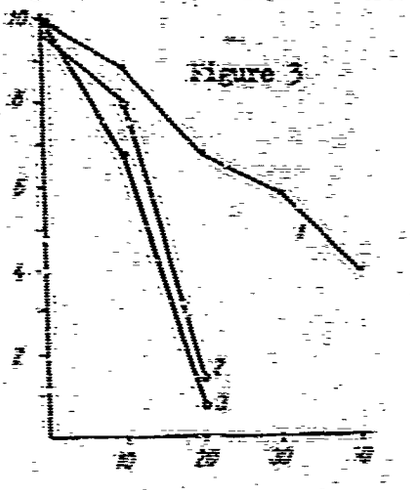


Figure 3



Inactivation of purified VEE virus at 50°.

1) Original virus in medium - 199 without serum
2) The same, diluted 5 times with physiological solution
3) Purified virus in medium - 199, diluted 5 fold by physiological solution.

Inactivation of purified virus in various salt solutions

1) Borax buffer, pH 8.8, with 12.5 magnesium sulphate.
2) Borax buffer, pH 8.8.
3) Physiological solution.

Ordinate axis - activity of the virus in lg ICE /ml
Abscissa - time (mins).

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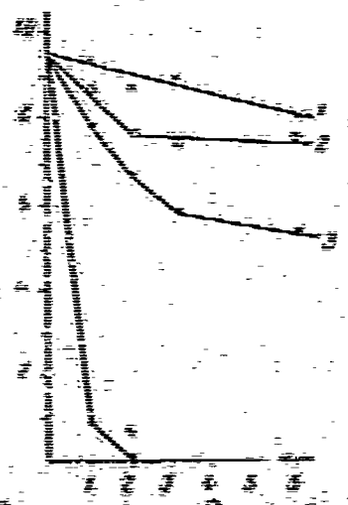
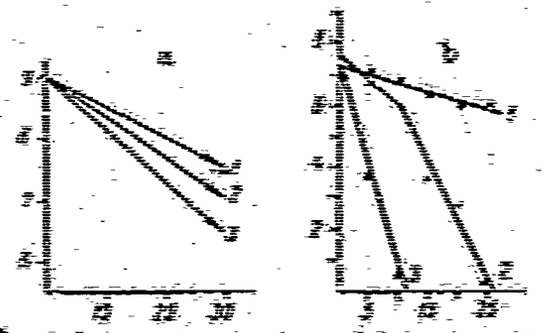
Figure 4 Inactivation of purified VSV virus with storage



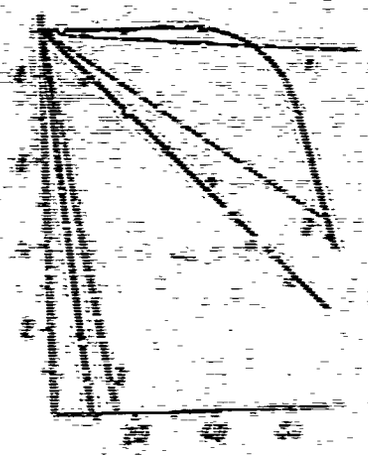
1 – at 5°; 2 – at –40°; 3 – at –80° with daily thawing and freezing; 4 – at 37°
Ordinate axis – virus activity in lg $TCID_{50}$/ml; abscissa axis – time of incubation (in days)

Figure 5 Inactivation of VSV virus with various values of pH at 37°



a) 1 – pH 9.0; 2 – pH 8.0; 3 – pH 7.0;
b) 1 – pH 5.0; 2 – pH 10.0; 3 – pH 6.0
Ordinate axis – virus activity in lg $TCID_{50}$ / ml; abscissa axis – time (in min.)

Figure 6 Inactivation of VSV virus with different temperatures in storage media, containing 0.1% glutamine.



1 – 20°; 2 – 34°; 3 – 50°. Control results are indicated by the dotted lines.
Along the ordinate axis – activity of virus in lg $TCID_{50}$ / ml, along the abscissa axis – time (in min.)

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buffer pH 9.0 (1:1) and aged at a temperature 37, 4 and -40°. Part of the trials, aged at -40°, we thawed only once, when after the appropriate date we established their infectious activity, the other part we subjected to daily thawing and freezing that considerably accelerated the dying off of the virus (Figure 4, curve 3). The virus at 37° was almost completely inactivated already on the following day.

At 4° the virus is more stable than at -40°, if the storage dates did not exceed 1 - 1½ weeks.

Effect of pH with the virus forming suspension on the thermal stability of the VEE virus.

On Figure 5a are presented graphs of the inactivation of the VEE virus at 53° and pH 7.0, 8.0, 9.0 during the first 20 mins. of heating. The increase of value of pH leads to a gradual increase of the speed of inactivation of the virus in these conditions.

Figure 5b demonstrates the inactivation of virus at 53° and values of pH 5.0, 10.0, and 4.0 ( curves 1,2 and 3 respectively). It is evident that such a large displacement of pH both in the alkaline and in the acid side significantly hastens the loss of infectious activity. Most rapidly perishes the virus in the strongly acid medium. It should be noted that the slope of curve 1 ("nuclein" type) is less than the more mildly sloping parts of curve 2, the slope of the abrupt part of which ("protein" type) in its turn is less than the slope of curve 3.

The effect of glutamine and some other additives to the virus containing suspension on the thermal stability of the VEE virus.

On Figure 6 are presented the dynamics of inactivation of the virus at 50 54 and 58° in medium # 199 with 2% bovine serum in the presence of 0.1% glutamine. The control data are denoted by the dashed line. At 54 and 58°, 0.1% glutimine somewhat accelerated the inactivation of the virus. At 50°, before the period of accelerated inactivation of the VEE virus beginning within 60 mins. of heating, it was determined as a stage of stabilization of the infectious titers. However the difference in comparison to the control is small and by the use of the titration method of platelets it cannot be acknowledged reliable.

In connection with that that we obtained similar results at 37°, and also for the determination of the effect on the stability of the virus of the combined presence of glutamine and magnesium sulphate, having a stabilizing effect on "protein" type of inactivation, there was supplied a special series of experiments.

The VEE virus we bred on TCE in Hrla solution with 0.22% bicarbonate of soda without serum, in order to avoid the effects of multiple components for the usually utilized composition of the storage medium. To the virus containing solution we added 0.1% glutamine, 12.5% magnesium sulphate, 0.1% bovine albumin and their combination. The virus we heated at 50° during 20 mins.



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The results of the experiments are presented in the Table. The greatest stabilizing effect was possessed by crystallized bovine albumin, this effect of it was somewhat reduced by the addition of magnesium sulphate. Magnesium sulphate somewhat stabilized the virus, in the same extent glutamin accelerated its inactivation. The combined introduction of 12.5% magnesium sluphate and 0.1% glutamin lead to appreciable acceleration of inactivation.

Table

Inactivation of VEE virus at 50° in media of various compositions[1]

| Composition of the medium in which are suspended the virus particles. | Reduction of infectiousness ( In lg BOE /ml) for 20 mins. of warming at 50° |
|---|---|
| Erla solution with 0.22% bicarbonate of soda | 2.8 |
| The same and 0.1% bovine albumin | 1.8 |
| The same and 0.1% glutamine | 3.1 |
| The same and 12.5% magnesium sulphate | 2.5 |
| The same and 0.1% bovine albumin and 12.5% magnesium sulphate | 2.3 |
| The same, 0.1% glutamine and 12.5% magnesium sulphate. | 4.6 |

1 Data the mean of the result of 3 parallel experiments.

## Discussion

The study of the course and of the thermodynamic parameters of the process of thermal inactivation of viruses leads to the conviction that the loss of infectious activity of the virions as a result of the effect of that or the other temperature, is derived, by a limited number of paths or procedures, from that corresponding to two basic mechanisms of inactivation - "nuclein" or "protein", that reflect the internal structure of the virus particles. Various external factors show diverse and regular effect on the probability of the development and the interrelationship of the indicated mechanisms of inactivation (references 6 and 7).



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An investigation of the curves of inactivation of infectious activity allows, to the extent determined, the judgement of the nature and [illegible] of the process. The determination of the dynamics of inactivation of the VEE virus with varied temperature and various conditions leads to the conclusion that the variation of the slope of one curve of inactivation in comparison with the others (for example, curves 1,2 and 3 on Figure 3), in certain limits, tells of the acceleration or retardation of the flow of the process with the maintainance of the mechanism or of the type of inactivation. On the other hand, an abrupt alteration of the slope of one curve in comparison with the others, established at one and the same temperature (curves 1 and 3 on Figure 3), and also the presence of a discontinuity on the curve (for example, curve 3 on Figure 2 or curve 2 on Figure 4), as a rule, show by the variation of the same mechanism of the process of thermal inactivation (References 2 - 4).

In the course of the inactivation the significant effect shows, apart from the structures of the components of the nucleocapside of the virus particles, their surface lipoprotein complex. The role of the surface of the lipoprotein complex, being formed with the immediate assistance of the cellular components (Reference 1), is displayed in various proportions to the heating at 50° of the virus populations, obtained on the cells of various origin. For the virus populations ($t^s$ variant), obtained on HeLa and HASKA cells, the speed of inactivation resulting, according to appearance, by the "protein" type, is increased in relation to the viruses from RES and TCE cells, being inactivated in these cases according to the "nuclein" type.

On the other hand, variations of thermal resistance of the $t^r$ and $t^s$ variants, obtained on TCE, are maintained with transformation to HeLa cells as to the system of producing the virus. But the temperature level, at which takes place a change of one ("nuclein") type with the other ("protein") (References 2,3), will be mixed in relation to the nature of the cells.

Thus, at least in application to the studied model, the increase of stability of the virus particles can take place owing to both increases of strength of the albumin structures of the nucleocapside (References 3,4), and the increases of stability of the lipoprotein complex, playing a protective role.

The properties of the surface of the lipoprotein complex establish the forming and duration of the period of induction - the initial period of retarded loss of infectious activity of the VEE virus, during which it is inactivated according to the "nuclein" type. Ultra-centrifuging of the virus substantially reduces its thermal stability, while, determined in a control experiment at 50°, the period of induction for the purified virus vanishes and the virus, at once, is inactivated according to the "protein" type. Simultaneously there grows the sensitivity of the VEE virus, in the usual conditions not sharply defined, expressed (Reference 2) to the thermally stabilizing effect of magnesium sulphate, reducing the speed of "protein" inactivation.

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Taking into account that in the process of ultra-centrifuging, with the purification of the VEE virus, there is observed its "loading", a variation of the floating density of the virus particles, resulting evidently owing to the plucking out of the lipoidal components (References 5,10), it is possible to postulate that the virus becomes more thermally sensitive by virtue of a disturbance of its lipoprotein complex.

This fact complicates the work with purified virus preparations, since the integrity of the surface lipoprotein complex has great value by the storage of the virus at low temperatures, particularly below 0° (Reference 11). If in the course of experimental work there is required frequent extraction of the suspension of the purified virus from the refrigerator, or if the duration of the work with it does not exceed 1 - 1½ weeks, on the basis of the data obtained by us, it is possible to recommend preferred storage at 4° in comparison to temperatures lower than 0°.

In a model of the virus of foot and mouth disease it was shown that displacements of pH show contrary effect on "protein" and "nuclein" type of inactivation (Reference 6). In a more acid medium "protein" inactivation was accelerated and "nuclein" - was retarded. In a more alkaline medium this relationship is reversed. As a result of the investigations conducted by us on the VEE virus, it is detected that variations of pH, in certain limits, do not change the duration of the period of induction and, consequently, do not influence the stability of the surface of the lipoprotein complex. In these conditions a decrease of pH reduces the speed of inactivation of the VEE virus of the "nuclein" type. A more coarse displacement of pH ( up to 6.0 or 10.0) shows destruction of the surface of the lipoprotein complex. and variation of the mechanism of inactivation of the virus.

From a number of tests of chemical additives to the virus containing suspension the greatest stabilizing effect was possessed by bovine albumin, it retarded the loss of infectious activity of the virus both according to the "nuclein" and the "protein" type. A sufficiently good effect was shown by the application of a complex of components, being contained in medium # 199.

According to our opinion, it is of definite interest to search for chemical substances showing selective effect on the inactivation of virus. If for the "protein" type such a selective effect is possessed by magnesium sulphate, then the specificity of the effect of glutamine on the "nuclein" type of inactivation is not successfully demonstrated reliably. Indirectly this fact can confirm the fact of neutralization of the stabilizing effect of magnesium sulphate by the addition of 0.1% glutamine. It should be taken into account that the chemical additives are effective, first of all, on the surface formation of the virions; the increase of stability of the surface lipoprotein complex does not change the speed of inactivation of the "nuclein" type, and only increases the time during which there is established precisely this type of inactivation. The effect on the "nuclein" loss of infectiousness is possible only with the conditions of sufficient penetration of the surface structures by the tested substance. But this increased penetrability may



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disturb the strength of the surface lipoprotein complex and may cause its premature destruction. Similar correlations are traced with the inactivation of VEE virus at 30° in the presence of glutamine (viz. Figure 6). The period of induction is considerably shortened but on the extent of it, in practise, there is not established inactivation according to the "nuclein" type.

Supplying the sum to the discussed, it is appropriate to note a series of general statements. The stability of the VEE virus at low temperatures is determined by the speed of its inactivation according to the "nuclein" type in the presence of sufficiently high temperatures, when the loss of infectiousness with the first minutes of warming is due to denaturation of the protein viruses, the thermal stability of the VEE virus is determined by the speed of inactivation by the "protein" type. In the interval between these two extreme cases, the stability of the virus depends, in the final analysis, on the duration of the induction period and, consequently, on that temperature condition when the period of induction will cease being established.

The studied biological, physical and chemical factors, influencing the process of thermal inactivation of the VEE virus, accelerated or retarded in different conditions the "nuclein" or "protein" type of inactivation of the virus. On the other hand, their effect was accomplished through the surface lipoprotein complex and involved the variation of the period of induction and also the temperature conditions at which the inactivation of the virus began to take place, principally according to the "protein" type.

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