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THERMOSTABILITY OF DISTEMPER VIRUS AND ITS NEUTRALIZATION BY ANTIBODY

By

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The techniques of tests for the detection of neutralizing antibody to distemper virus (DV) have varied greatly (9), in particular with respect to the time and temperature chosen for incubation of the virus-serum mixture. However, since DV is relatively heat labile, it is important to conduct the neutralization test at a temperature where thermal inactivation of the virus is minimal while the reaction between virus and antibody proceeds at a reasonably high rate. In view of this, it is surprising that no quantitative data related to thermal inactivation of DV are available.

The rate at which DV was inactivated at various temperatures (5, 25, 37, 56°C) was therefore studied in order to select a temperature at which thermal inactivation of DV was slow, while the association rate for the reaction between virus and neutralizing antibody proceeded at an adequately rapid rate.

For the detection of small amounts of antibody, it is important that the reaction between virus and antibody is allowed to

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reach equilibrium ("steady state") prior to assessing the mixture of the two reactants for free virus. The time required for virus-antibody mixtures to reach such a reversible equilibrium state was determined at indicated temperatures.

MATERIALS AND METHODS

Embryonated hens' eggs. Fertile White Leghorn eggs, supplied from a single hatchery, were incubated at 37°C for 7 days. The eggs were then candled to mark the position of the embryo and the air sac. Fertile eggs were inoculated by the stab method (8) with 0.2 ml inoculum per egg. After an additional 7 days incubation, chorioallantoic membranes (CAMs) were examined for DV lesions in a Quebec colony counter. A membrane which showed two or more greyish-white opaque pocks was considered positive for DV infection.

Virus. The Wisconsin FXNO isolate of DV was used and the diluent was 0.01 M phosphate buffer of pH 7.4, and containing 5 % glycerine, 50 µg streptomycin sulphate, 250 i.u. penicillin and 50 units bacitracin per ml. In the present study, the sixty-fifth to seventieth egg passage levels were used. The virus stock was prepared by inoculating eggs with 200 to 400 EID_{50}. A 1:2 dilution of pooled infected CAMs was prepared in phosphate buffer and ground in a glass grinder. The tissue suspension was clarified by centrifugation and the supernatant was stored at —60°C.

Sera. Five silver foxes were vaccinated with 1000 ferret LD_{50} of Green's Distemperoid virus at 10 weeks of age and bled 30 days later. The sera were collected, pooled and stored at —20°C without preservatives. The sera were inactivated at 56°C for 30 minutes immediately before use.

Virus titrations. The virus was diluted in six to seven 2-fold increments in chilled phosphate buffer and eight to ten eggs were inoculated with each virus dilution.

Calculation of virus and serum titers. Dead embryos were not included in the calculation of the 50 % endpoints. The method of *Reed & Muench* (10) was used for the calculation of the virus and serum titers. Virus titers were expressed in $\log_{10}$ 50 % egg infectious doses (EID_{50}) per 0.2 ml of undiluted CAM homogenate. Serum titers were expressed as the negative $\log_{10}$ serum dilution which protected 50 % of the CAMs from virus infection.

Temperature of water baths. The temperatures of the water baths varied as follows: 55°—56°C; 36°—37°C; 24°—25°C; and 3°—7°C. All water baths were covered to prevent heat loss throughout the experiment. The 56°C and 37°C water baths were equipped with a motor-driven propeller.

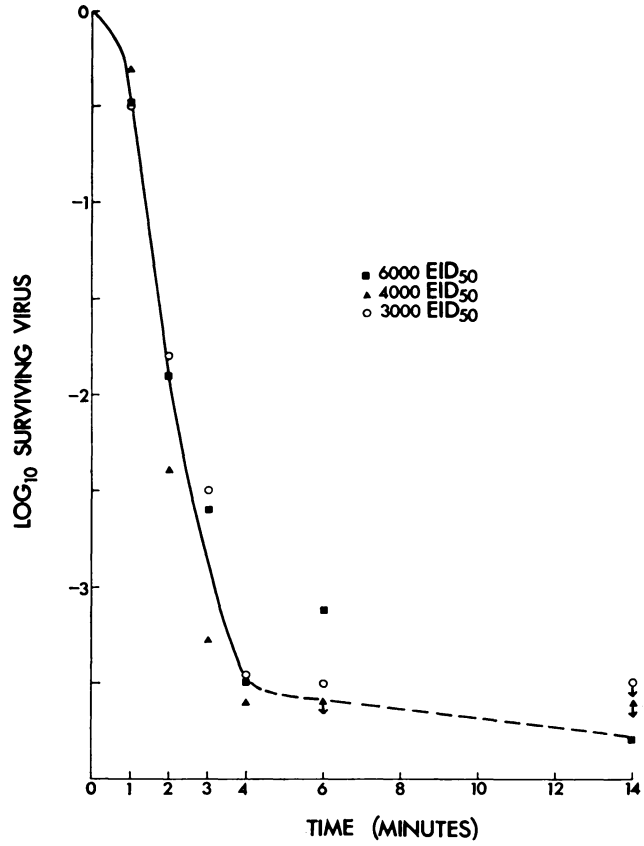
EXPERIMENTS AND RESULTS

Thermal inactivation of distemper virus.

The rate of thermal inactivation of DV was studied at four different temperatures: 5, 25, 37 and 56°C. In these experiments the virus suspension was titrated just prior to its exposure to the selected temperature (0 time). Timing began immediately after the tubes containing DV were immersed in the water bath. At each sampling time a DV tube was shaken and a quantity of virus removed for virus infectivity assay. The withdrawn sample was immediately diluted in chilled phosphate buffer and kept in an ice bath during inoculation.

Thermal inactivation at 56°C. The results of preliminary titrations at 56°C indicated that inactivation proceeded very rapidly within 6 minutes. Virus samples, therefore, were collected after 0, 1, 2, 3, 4, 6 and 14 minutes heating. At 56°C thermal equilibrium of the virus sample was reached in $\simeq 1\frac{1}{2}$ minutes after the sample was placed in the water bath. The amount of virus used varied between 3000 and 6000 EID_{50} in different experiments. In a typical experiment 1 ml of DV was pipetted into each of seven tubes which were held in an ice bath until the test began. At each sampling time one tube was removed from the water bath, immediately immersed in ice and the virus suspension diluted 1:2 in chilled phosphate buffer. At the end of the 14-minute period the dilutions were completed and the eggs inoculated.

Regressions of inactivated DV on time were computed for each experiment. The points at 1 minute (Fig. 1) possibly suggest a shoulder, but this could be due to the fact that the virus sample required about $1\frac{1}{2}$ minutes to reach thermal equilibrium at 56°C. All regressions were linear, negative and significant (Table 1). The smallest and largest regression coefficients ($b = -0.88$ and $b = -1.2$) were not significantly different ($t = 0.62$, $P > 0.5$) and it was concluded that the difference in initial DV amount had no detectable effect on the rate of DV inactivation at 56°C.



F i g. 1. Thermal inactivation of distemper virus in 0.01 M phosphate-glycerine buffer at 56°.

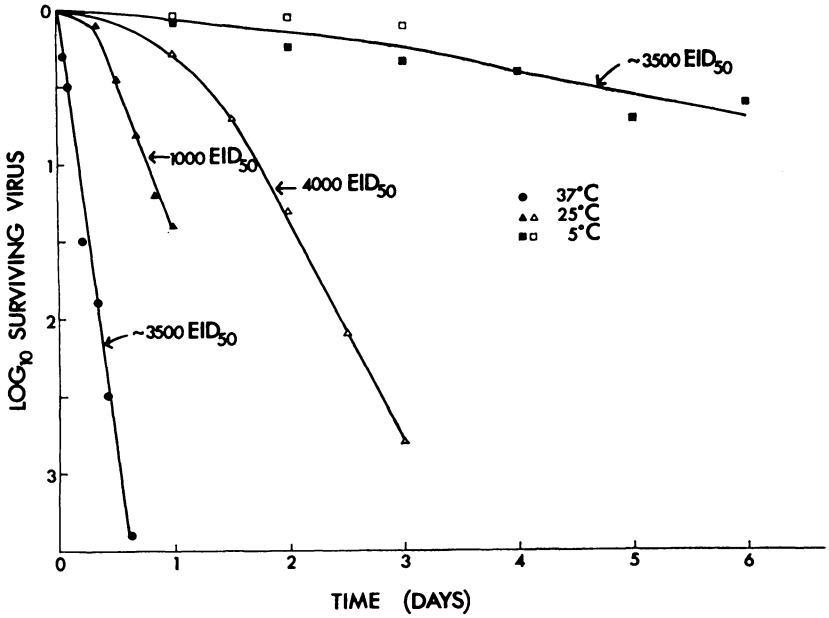
T a b l e 1. Thermal inactivation of distemper virus: linear regressions and F values for linearity.

Temperature of inactivation	Initial DV amount (EID_{50})	Linear regressions	F values
56°	≃ 6000	Y = 0.15 — 0.92X	186, P<0.001
	≃ 4000	Y = 0.32 — 1.2X	24, 0.025<P<0.050
	≃ 3000	Y = 0.12 — 0.88X	62, 0.010<P<0.025
37°	≃ 3000	Y = 0.04 — 0.23X	1900, P<0.001
	≃ 4000	Y = 0.11 — 0.23X	175, 0.005<P<0.010
25°	≃ 1000	Y = 0.56 — 0.085X	568, P<0.001
	≃ 4000	Y = 2.54 — 0.053X	322, P<0.001

The combined linear regression was also linear, negative and significant ($Y = 0.20 - 1.01X$, $F = 41$, $0.010 < P < 0.025$).

The half-life of the surviving virus at 56°C was $\sim$ ½ minute (Fig. 1). After about 4 minutes the curve appeared to bend abruptly and the inactivation thereafter to proceed at a much slower rate.

Thermal inactivation at 37°C. The results of preliminary titrations indicated that approximately 0.5, 2.5 and 3.5 $\log_{10}$ EID_{50} virus was inactivated in 1, 12 and 24 hours, respectively.



F i g. 2. Thermal inactivation of distemper virus in 0.01 M phosphate-glycerine buffer at 37°, 25° and 5°C.

The virus was kept in one stoppered tube. Two different sampling time series were used in the experiments. In the first two experiments, DV was titrated at 0, 5, 10 and 15 hours; in the subsequent three experiments, at 0, 1, 2 and 8 hours (Fig. 2). The amount of virus used varied between 3000 and 4000 EID_{50}.

The $\log_{10}$ surviving virus for the two series of experiments appeared to follow a linear relationship with time (Table 1). No difference was found between the two regression coefficients ($t = 0.01$, $P > 0.5$). The combined slope was also linear, negative and significant ($Y = 0.09 - 0.23X$, $F = 572$, $P < 0.001$) (Fig. 2). The half-life of the surviving virus was about 1 hour.

Thermal inactivation at 25°C. The results of preliminary titrations indicated that $\simeq$ 0.3 $\log_{10}$ EID_{50} of virus was inactivated within 12 hours and about 1.5 $\log_{10}$ EID_{50} in 48 hours.

Distemper virus was kept in a stoppered tube. A first experiment was performed using 4000 EID_{50} of virus at sampling times of 0, 24, 36, 48, 60 and 72 hours and a second experiment with 1000 EID_{50} of virus at sampling times of 0, 8, 12, 16, 20 and 24 hours.

Regressions of inactivated virus on time were computed for the two experiments, omitting the 0 time from both regressions. The two regressions were linear, negative and significant (Table 1). A significant difference was found between the two regression coefficients ($t = 4.5$, $0.001 < P < 0.01$).

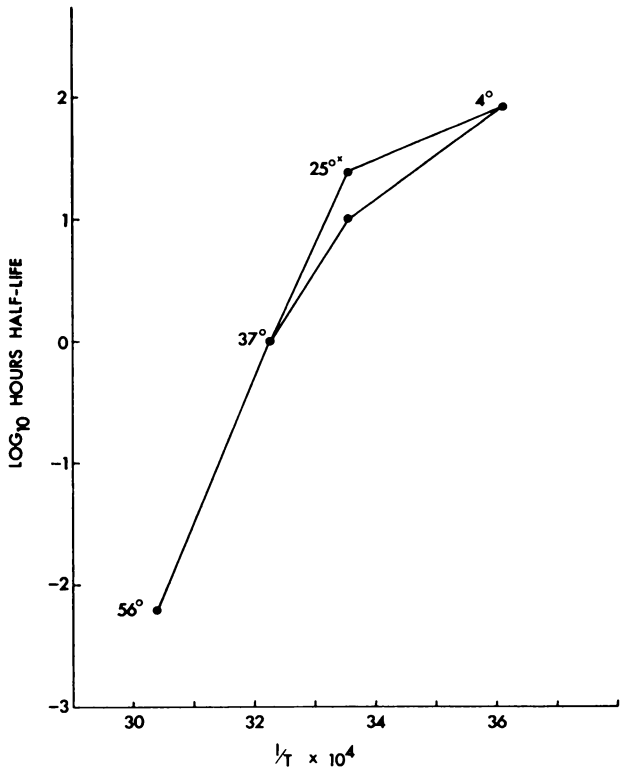
The inactivation curves for both virus quantities showed an initial shoulder (Fig. 2). Thereafter, virus was inactivated according to first order kinetics. Since the shoulder on the inactivation curve was more marked when the higher virus input (4000 EID_{50}) was used, the initial half-lives differed considerably in the two experiments. The half-lives were approximately 10 (1000 EID_{50}) and 26 hours (4000 EID_{50}), respectively.

Thermal inactivation at 5°C. The virus was titrated in 24-hour intervals. In the first series of experiments sampling times were 0, 1, 2, 3, 4, 5 and 6 days, and the amount of virus used was about 3000 EID_{50}. In the second series of experiments samples were taken at 0, 1, 2 and 3 days with about 4000 EID_{50} of virus employed.

Since the points of the two series of experiments were fairly scattered (Fig. 2), an enumerative rather than a quantal assay was applied to the percentages of the mean surviving virus. No true differences in occurrence were observed between the two series of experiments ($\chi^2 = 7.1$, $0.050 < P < 0.100$). Therefore, the results of the two experiments were combined to calculate a linear regression. The regression was linear, negative and significant ($Y = 0.06 - 0.12X$, $F = 45$, $0.001 < P < 0.005$).

It is hard to determine whether virus is inactivated according to first order kinetics at this temperature since the rate of inactivation is slow and the results show considerable variation. The half-life of DV at 5°C was 3 to 4 days. At the end of 6 days about 25 % DV infectivity remained (Fig. 2).

A single straight line relationship could not be demonstrated with the Arrhenius plot (7). The plotting of half-lives for ad-



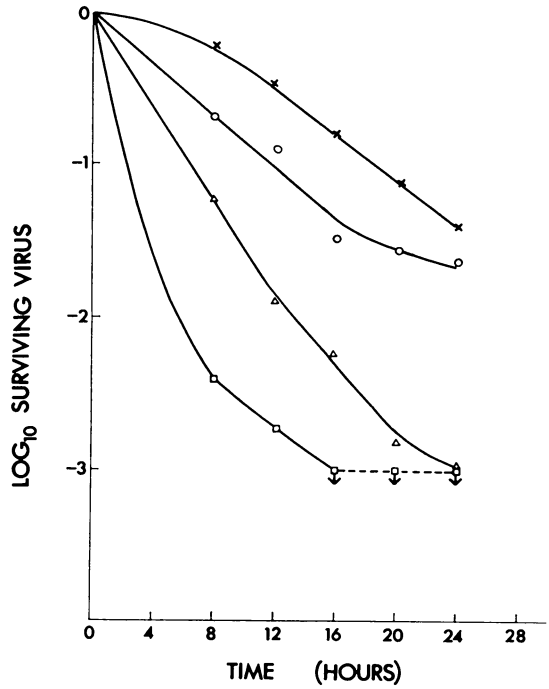
F i g. 3. Arrhenius plot of thermal data for inactivation of distemper virus. Logarithms of half-lives versus reciprocals of absolute temperatures. × The longer half-life was obtained with an initial virus dose of 4000 EID_{50}, the shorter with 1000 EID_{50}.

ditional temperatures would be necessary to determine accurately the deflection point(s) on the curve in Fig. 3. Studies of the thermal inactivation of other viruses (1, 3, 4, 11) have also failed to demonstrate a rectilinear relationship in the Arrhenius plot.

The thermal inactivation studies were carried out to find a temperature where inactivation proceeded at a slow rate, yet allowing the reaction between virus and antibody to reach approximate equilibrium in a time period that would be convenient for neutralization tests. It was evident that such tests could not be performed at 56°C or 37°C since thermal inactivation of DV was too rapid at these temperatures. Thus, in the next series of experiments the time required for the reaction between DV and small amounts of antibody to reach equilibrium at 25 and 5°C was determined.

Kinetics of neutralization of distemper virus by antibody.

Kinetics of neutralization at 25°C. Distemper virus ($\simeq$ 1000 EID_{50}) was mixed in equal volume with dilutions of pooled immune fox serum ($10^{-3.0}$, $10^{-3.3}$ and $10^{-3.7}$) and with buffer as a control. The reaction mixtures were incubated at 25°C with sampling times at 0, 8, 12, 16, 20 and 24 hours. Removed samples were placed in an ice bath, serial 2-fold dilutions prepared with



F i g. 4. Kinetics of neutralization of distemper virus by antibody at 25°C. × distemper virus + buffer, o distemper virus + immune serum $10^{-3.7}$, Δ distemper virus + immune serum $10^{-3.3}$, □ distemper virus + immune serum $10^{-3.0}$.

chilled buffer and the dilutions immediately inoculated into embryonated eggs, using nine eggs per dilution.

Significant neutralization was obtained with the $10^{-3.0}$ and $10^{-3.3}$ serum dilutions (Fig. 4). The reaction between DV and antibody in the $10^{-3.0}$ sample reached equilibrium within about 8 hours. For the $10^{-3.3}$ dilution, 16 to 20 hours appeared to be required.

Kinetics of neutralization at 5°C. The serum-virus mixtures and buffer-virus mixtures were the same as in the 25°C experi-

ment, except that serum dilution $10^{-4.0}$ also was included. The reaction mixtures were sampled and assayed as described above. The sampling times were 0, 1, 2 and 3 days.

Like in the experiments at 25°C only the two lowest serum dilutions ($10^{-3.0}$, $10^{-3.3}$) showed demonstrable virus neutralization (Fig. 5). The reaction between antibody in the $10^{-3.0}$ sample and virus appeared to have reached "steady state" within 2 days. For the $10^{-3.3}$ dilution more than 3 days seemed to be required.

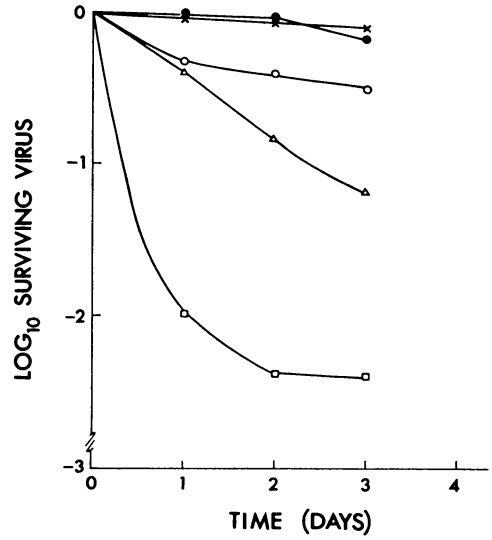


Fig. 5. Kinetics of neutralization of distemper virus by antibody at 5°C. × distemper virus + buffer, • distemper virus + immune serum $10^{-4.0}$, o distemper virus + immune serum $10^{-3.7}$, Δ distemper virus + immune serum $10^{3.3}$, □ distemper virus + immune serum $10^{-3.0}$.

Since the results at 5°C indicated that ≥ 2 days were needed to detect small amounts of antibody, this was considered, from the practical standpoint, too long an incubation time to be employed in routine neutralization tests. Instead, 16 to 24 hours incubation at 25°C appeared to be an adequate and convenient incubation condition for use.

DISCUSSION

Distemper virus is a relatively heat labile virus. In neutralization tests thermal inactivation of DV must be carefully controlled so that loss of virus infectivity due to heat is not con-

sidered due to neutralization by antibody. A temperature must be used where heat inactivation is moderate, while the reaction between virus and even small amounts of antibody proceeds at a reasonably high rate.

The half-life of DV at 56°C in this study was approximately ½ minute, whereas *Bussell & Karzon* (2) estimated the half-life to be 2 to 3 minutes, using the plaque assay technique. Thus, loss of infectivity at this temperature was too rapid to be considered for neutralization tests. After about 4 minutes' inactivation the curve bends abruptly to a plateau, possibly indicating a thermal resistant virus population, which however would constitute less than 0.1 per cent of the virus population.

A possible temperature considered for use in neutralization tests with distemper virus was 37°C, since for other myxoviruses such as Newcastle and influenza A, the half-life at this temperature is about 24 hours (6). However, the half-life of DV at 37°C was only about 1 hour which agrees with *Bussell & Karzon*'s results. Consequently, the heat lability of DV precluded the use of this temperature in neutralization tests designed to detect minor amounts of antibody.

In the 25°C study thermal inactivation proceeded at different rates, depending upon the initial amount of virus used. Thus, inactivation of $\simeq$ 1000 EID_{50} after 8 hours proceeded at a more rapid rate than of the $\simeq$ 4000 EID_{50} after 24 hours. This difference was probably related to a less protective effect from extraneous protein in the more dilute virus preparation (1000 EID_{50}).

Another temperature that was considered for use in neutralization tests was 5°C. *Bussell & Karzon*'s work revealed a half-life of 2 weeks at 4°C. In contrast, the half-life of DV in this study at about 5°C was as short as 3 to 4 days; however, points at this temperature were too scattered to allow a reliable estimation of the half-life.

The thermal inactivation investigations indicated that it would be impracticable to conduct the neutralization test at temperatures exceeding 25°C. Furthermore, since at least 2 to 3 days were required for the neutralization reaction to reach equilibrium at 5°C, this temperature was not considered suitable for routine use. *Gard* (5) observed that the reaction between Theiler's virus and homologous neutralizing antibody needed about 10 days to reach equilibrium at 4°C. It would appear that 16 hours at 25°C

is an adequate incubation condition for DV-serum mixtures, and that this would provide a reasonably sensitive test for neutralizing antibody.

REFERENCES

1. *Bachrach, H. L., S. S. Breese, Jr., J. J. Callis, W. R. Hess & R. E. Patty:* Inactivation of foot-and-mouth disease virus by pH and temperature changes and formaldehyde. Proc. Soc. exp. Biol. (N.Y.) 1957, *95*, 147—152.
2. *Bussell, R. H. & D. T. Karzon:* Canine distemper virus in chick embryo cell culture (plaque assay, growth and stability). Virology 1962, *18*, 589—600.
3. *Dougherty, R. M.:* Heat inactivation of Rous sarcoma virus. Virology 1961, *14*, 371—372.
4. *French, C. R. & R. E. Armstrong:* Thermal inactivation of MEF 1 poliovirus. Canad. J. Microbiol. 1960, *6*, 175—181.
5. *Gard, S.:* Neutralization of Theiler's virus. Acta path. microbiol. scand. 1955, *37*, 21—30.
6. *Ginsberg, H. S. & F. L. Horsfall, Jr.:* Concurrent infection with influenza virus and mumps virus or pneumonia virus of mice (PVM) as bearing on the inhibition of virus multiplication by bacterial polysaccharides. J. exp. Med. 1949, *89*, 37—52.
7. *Glasstone, S.:* Textbook of Physical Chemistry, 2nd ed., D. Van Nostrand Co., New York 1946.
8. *Gorham, J. R.:* A simple technique for the inoculation of the chorioallantoic membrane of chicken embryos. Amer. J. vet. Res. 1957, *18*, 691—692.
9. *Lo, J. P., W. M. Dickson & J. R. Gorham:* Errors in distemper virus titrations performed in embryonated eggs. Arch. ges. Virusforsch. 1964, *15*, 74—90.
10. *Reed, L. J. & H. Muench:* A simple method of estimating 50 per cent end points. Amer. J. Hyg. 1938, *27*, 493—497.
11. *Svehag, S.-E.:* Thermal inactivation of blue tongue virus. Arch. ges. Virusforsch. 1963, *13*, 499—510.

SUMMARY

The thermostability of distemper virus in a phosphate-glycerine buffer of pH 7.4 was studied at various temperatures.

Thermal inactivation curves suggested first order kinetics except at 25°C where the curves exhibited initial shoulders. The estimated half-life for distemper virus at 5, 25, 37 and 56°C were 3—4 days, 10 hours, 1 hour and ½ minute respectively. A single straight line relationship could not be demonstrated in the Arrhenius plot.

Approximate equilibrium for the reaction between distemper virus and small amounts of neutralizing antibody was established at 5°C in 2 to 3 days and at 25°C in 16—20 hours. It was concluded that 16 hours

at 25°C provided an adequate and convenient incubation for the virus antibody reaction in a sensitive test for distemper virus neutralizing antibody.

ZUSAMMENFASSUNG

Der Hundestaupe Virus: seine Wärmestabilität und die Neutralisierung von den Antikörpern.

Die Wärmestabilität des Hundestaupe Virus war in einem Phosphat-Gyzerin Puffer von pH 7,4 bei verschiedenen Temperaturen studiert worden.

Die Kurven der Wärmeinaktivierung erschienen bei erstem Grade Kinetik, ausser bei 25°C, wo die Anfangsinaktivierung von dem ersten Grade Reaktion abweichte Die Halbierungszeiten für den Hundestaupe Virus bei 5, 25, 37 und 56°C waren auf 3—4 Tage, 10 Stunden, 1 Stunde und ½ Minute geschätzt worden. Einen einfachen, geradlinigen Zusammenhang konnte man in dem Arrhenius „plot" nicht bekommen.

Die Reaktion zwischen dem Hundestaupe Virus und geringen Mengen neutralisierender Antikörper, erreichte ungefähr das Gleichgewicht bei 5°C in 2 bis 3 Tagen, und bei 25°C in 16 bis 20 Stunden.

Eine empfindliche Untersuchung, neutralisierender Antikörper gegen Hundestaupe Virus, gewährte eine Inkubationszeit von 16 Stunden bei 25°C.

SAMMANFATTNING

Valpsjukevirus: dess värmestabilitet och neutralisering medelst antikroppar.

Värmestabiliteten hos valpsjukevirus studerades vid olika temperaturer i en fosfat-glycerin buffert av pH 7.4.

Värme-inaktiveringskurvorna förehöll uppvisa första graden kinetik utom vid 25°C där den initiala inaktiveringen avvek från en första grads reaktion. Halveringstiderna för valpsjukevirus vid 5, 25, 37 och 56°C uppskattades till 3—4 dagar, 10 timmar, 1 timme och ½ minut respektive. Ett enkelt rätlinjigt samband erhölls ej i Arrhenius „plot".

Reaktionen mellan valpsjukevirus och små mängder neutraliserande antikroppar uppnådde ungefärlig jämvikt vid 5°C inom 2 till 3 dagar och vid 25°C inom 16 till 20 timmar. 16 timmar vid 25°C erbjöd ett adekvat och lämpligt inkuberingsförfarande för virus-antikropps reaktionen i en känslig test för neutraliserande antikroppar.

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