THERMAL DECAY KINETICS OF COLORADO TICK FEVER VIRUS AND THE EFFECTS OF VARIOUS DILUENT SOLUTIONS ON VIRUS HEAT INACTIVATION

by

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INTRODUCTION

A. Statement of Problem

This investigation was prompted by the obvious need for safe and effective vaccines specific for certain members of the arthropod-borne virus (arbovirus) group. The current first line of defense against these agents is composed of vector control measures of varying (and frequently dubious) effectiveness (1). For use against relatively few members there are available vaccines of either the killed or live attenuated virus type; these are, for the most part, formalinized mouse brain or cell culture preparations containing relatively large amounts of nonviral host material. The autoimmunizing ("encephalitogenic") capability of vaccines incorporating mouse brain-derived virus has been known for some 35 years (2); extensive use of such preparations is therefore currently limited to certain veterinary applications. While advances in cell culture methodology in recent years have permitted the formulation of a variety of arbovirus vaccines derived from infected cell lysates, maintenance of adequate vaccine potency under practical storage conditions has proven difficult (3,4,5,6).

The ideal virus vaccine, whether of the attenuated or inactivated type, is one which incorporates serologically stable virus substantially free of any potentially autoimmunizing antigens or overtly toxic components. The production of such a vaccine requires a more or less purified virus that is sufficiently stable as to retain its antigenic

character throughout vaccine preparation, storage and administration. In those cases where the agent is inherently heat-labile, diluents must be sought which, while physiologically inert in themselves, serve to stabilize the virus throughout the purification, preparation and handling procedures.

Little is known of the response of arboviruses to physical and chemical agents. The lack of critical analytical data concerning the basic thermal stability of the virus of Colorado tick fever typifies the void in our understanding of this virus group. The lag in arbovirus vaccine development is in part the result of lack of available efficient purification methods for the group as a whole. Evolution of such methods is largely contingent upon definition of the response of group members to such environmental factors as elevated temperature and specific diluents. This study was therefore aimed at 1) a basic exploration of the thermal stability of mouse brain preparations of Colorado tick fever virus; and 2) attempts to modify virus stability (or the lack of it) by treatment with a variety of ionic diluents.

B. The Effects of Physical and Chemical Agents on Viruses

The effect of any physical or chemical agent on a virus must ultimately depend upon its ability to provoke a structural change in one or more of the viral constituents. Such an alteration may be manifested as a change, usually a decrease, in the number of measurable infective units, or titer; a modification of the chemical composition of the particle; or a change, frequently subtle, in the physical

properties of the virion[1] or viral population. Infectivity, for example, may decrease either because of a direct alteration in the viral genome leading to the inability of the virus to direct the production of normal progeny, or because of superficial damage to the outer layers of the virion resulting in failure of the particle to attach to specific host cells, or to penetrate, or to initiate the first stages of replication within the cell. The site of action or target of the agent may, therefore, be the nucleic acid, the viral protein capsid or, when present, the envelope or any of its attendant surface structures.

It is known that different environmental agents inactivate or otherwise alter virus particles by diverse, yet highly specific mechanisms (8,9). Strictly physical methods include such mechanical treatments as sonic oscillation and shaking, as well as exposure to ionizing (X-, alpha and other heavy particle) and nonionizing (near and far ultraviolet and visible) irradiation. Inactivation due to decay of incorporated radioactive phosphorus, while operationally not a physical treatment, is usually grouped with the other types of irradiation because of the similarity of the inactivation process. All strictly physical methods of virus inactivation are thought to function via essentially the same overall mechanism; i.e., disruption of the continuity of the polynucleotide chain by simple scission.

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[1]The nomenclature used throughout this thesis is that proposed by Caspar, Dulbecco, Klug, Lwoff, Stoker, Tournier and Wildy (7).

Chemical agents influencing viral stability include formaldehyde; protein-denaturing, oxidizing and alkylating agents; organic solvents; enzymes; and a variety of miscellaneous substances such as heavy metals and other protein-precipitating agents. Formaldehyde appears to react principally with the amino and ribose (and perhaps other non-ionized) groups of the viral nucleic acid, rather than with the relatively more ionic amino and imino groups of capsid protein. Protein-denaturing agents, such as anionic detergents, urea and guanidine, act (as one might expect) primarily by disintegration of the protective capsid and envelope material, frequently releasing into the surroundings intact and often infective nucleic acid. Oxidizing and alkylating agents (other than peroxides) probably inactivate or alter virus by inducing gross changes in base composition of the nucleic acids. The function of peroxides, on the other hand, is thought to be related to oxidative changes at the level of the capsid. While only a few viruses are wholly inactivated by proteolytic enzymes, exposure to such enzymes frequently results in drastic changes in serological reactivity and electrophoretic mobility. Organic solvents represent a group of heterogeneous substances, not only chemically but with regard to mode of action against viruses. Most will denature capsid protein, particularly under conditions of elevated temperature. But at low temperatures several organic solvents serve to precipitate viruses with intact capsids and little or no loss of infectivity; thus such solvents as methanol and ethanol have been used extensively for purification purposes. Longer chain (and therefore less soluble) alcohols, such as

butanol, have been used as well for the early steps of purification of crude virus suspensions. Nonpolar lipid solvents, such as chloroform-amyl alcohol mixtures and certain fluorocarbons, which act by denaturing protein at solvent-water interfaces, have been used with particular success for deproteination and purification of suspensions containing viruses free from essential lipid. Diethyl ether is of interest because of the relatively specific nature of its action. It apparently has no effect whatever on lipid-free viruses, while inactivating to various degrees those containing lipid as an essential component. An exception is the virus of Western equine encephalitis, which though rich in lipid content (54% by weight) is impervious to the presence of ether. While the action of diethyl ether is clearly directed at the lipid constituent of a virus, the net effect is one of rupture of the virion and release of the inner capsid and nucleic acid in various stages of aggregation. Infectivity is quickly lost as a result of this treatment. The effect of protein-precipitating agents, such as safranine, tannic acids and heavy metals, appears to be more or less related to simple physical "clearing" of the virions from the suspension, although there are conflicting views in this regard. At any rate, the effect is seen to be largely reversible in most cases by such techniques as dialysis, treatment with alcohols, picric acid, acetone or reducing agents.

We have thus far considered only the effect of physical and chemical agents upon viruses. There is a third group which has been termed, for want of a better name, the "physicochemical agents"

affecting virus stability. While a great deal of information is available concerning their gross effects upon specific viruses, they are as a group probably less well understood concerning their site of action than either the physical or chemical agents. Examples of these physicochemical factors include high temperatures, desiccation, variations in pH, and salt concentration both as a function and independent of the osmotic environment. Though the means by which these factors influence viral stability is unclear in most cases, it should be apparent that they are very closely interrelated. A change in one is likely to bring about a related change in another. For example, a change in the salt concentration of a virus suspension may influence the pH as well as the degree of hydration of viral components. Many viruses are susceptible to osmotic shock to a degree resembling that of a bacterium; the implication is that the outer covering of the virion is less permeable to solute than to water. Phage have been particularly well studied in this regard. Dilution of a suspension of one of the T-even phage from a concentrated NaCl (or other ionic or nonionic) solution into distilled water causes leakage of DNA from the virion and a subsequent drastic and irretrievable loss of infectivity of the suspension. Repeated cycles of freezing and thawing are known to inactivate most viruses, the extent of the inactivation determined mainly by the working diluent. It is difficult to study and control such manipulations since the inactivation observed may depend upon rather transient conditions occurring only at the moment of ice crystal formation (freezing) and destruction (thawing). Although rapid

changes in temperature probably contribute to the adverse effect, the formation of ice crystals and the resulting high local solute concentrations are probably the principal cause of the inactivation observed. A frozen virus preparation is similar in substance to a dried one in that both are effectively desiccated; the first by isolation of the water in the form of ice crystals, the second by sublimation or evaporation. Some viruses, such as phage T_1 and the small RNA phages, withstand drying at room temperature particularly well. Others, such as the large, complex animal viruses, tolerate the procedure very poorly. Storage in medium containing serum or glycerol at very low temperatures is probably the most effective means of preserving virus infectivity.

Another of the physicochemical factors, heat, is the subject of the remainder of this introductory discussion.

C. The Effect of Heat on Viruses and Viral Components

A virus is a large and extremely complex molecular structure. It owes its ability to function (i.e., to successfully parasitize a living cell) to its inheritance of a precise chemical configuration. The infective configuration of a virus is not ultimately stable. For any known virus, regardless of its apparent resistance to denaturation, there exists a different, though perhaps similar, configuration having a greater stability or lower potential energy. The heat denaturation of a virus or any other biological substance can be thought of as a process of changing its configuration via exposure to increased temperature from the inherited or normal form to one of perhaps greater

ultimate stability but no longer of the precise orientation for its proper function (10).

A virus, of course, is not simply a single molecular species, but an aggregate of molecules each of which has a function or plays a part in some function. The loss of normal configuration of one of these molecules may or may not influence the activity of the virus that we observe; this is entirely dependent upon the particular activity that we are measuring, be it infectivity for tissue culture cells, electrophoretic mobility or sedimentation velocity. The most convenient activity to measure, and therefore the most frequently studied, is that of infectivity. Other virus characters are less frequently studied.

In preparations containing considerable amounts of complex organic material such as serum or cell extracts, most viruses are quite stable, losing little if any infectivity even at room temperature over prolonged periods of time. Inactivation is usually slight in such preparations until temperatures of the order of 50 C or more are attained. Even at relatively high temperatures there are wide variations in the rate of thermal decay of different viruses. The extremes are represented by tobacco mosaic and southern bean mosaic viruses, which are practically stable at 65 C, becoming inactivated to any appreciable extent only around 75 C (11), and the parainfluenza viruses, especially the respiratory syncytial virus, in which thermal decay can be detected even when stored in complex organic media at temperatures as low as -40 C (12). In general, the ether-sensitive animal viruses (those containing essential lipid) are among the most heat-labile while certain

of the plant viruses are the most refractory to the effects of heat (9).

If a virus preparation is exposed to a temperature sufficient to cause inactivation (as by immersion in a waterbath) and aliquots are withdrawn at intervals and assayed for infectivity, the data obtained can be shown to fulfill the requirements of a first-order reaction (13). In general terms, a first-order or unimolecular reaction is one in which the rate of reaction is directly proportional to the concentration of a single reacting substance (14). If such data are plotted on semi-log paper in which the ordinate is the logarithm of the infectivity and the abscissa is time, the result is a straight line, provided that a sufficient number of samples have been assayed. The initial rate of inactivation of viruses at high temperature is therefore exponential. The ratio of virus activity V to the initial activity V_0 decreases with increasing time t of exposure, according to the equation:

$$\frac{V}{V_0} = e^{-kt} \qquad \text{(Eq. 1)}$$

where k is a temperature-dependent rate constant. In this case, the first-order exponential inactivation by heat indicates that a constant fraction of the virions undergoes a denaturing structural change in each unit of time, and that only one such change is required to inactivate a given particle.

The same concept can be expressed in a somewhat different form by:

$$\frac{-dc}{dt} = kc \qquad \text{(Eq. 2)}$$

where c is the concentration of the reactant (virus titer) at time t, and k, once again, is the rate or velocity constant which is specific for the reaction at any definite temperature. The negative sign here indicates that c decreases while t increases. It is evident from Equation 2 that the reaction rate at any instant is easily obtained by simply multiplying the constant k by the concentration of the reactant at that instant. For practical use, Equation 2 is rearranged to:

$$-dc/c = kdt$$

and then integrated; thus if C_0 is the initial virus concentration and C is the concentration at any subsequent time t, then:

$$k = \frac{1}{t} \ln \frac{C_0}{C} \qquad \text{(Eq. 3)}$$

and by converting to logarithms of the base 10:

$$k = \frac{2.303}{t} \qquad \frac{C_0}{C} \qquad \text{(Eq. 4)}$$

Equation 4 is known as the kinetic equation for a reaction of the first order; it provides a practical means for calculating the specific reaction rate of the inactivating process at a definite temperature. The velocity constant, k, is a number per unit of time and may be expressed in reciprocal seconds or other units. When, for example, k has a value of 0.001 sec^{-1}, the virus titer is decreasing at the rate of 0.1% per second. Knowledge of the specific reaction rate constant enables one to calculate the amount of material which will react in a given time,

or the time required for any specific portion of the material to react (15). For example, the period of half life ($t_{\frac{1}{2}}$) of a viral suspension exposed to heat can be projected by simply "plugging" the proper values into the first-order kinetic equation, Equation 4, as follows:

$$k = \frac{2.303}{t_{\frac{1}{2}}} \log \frac{1}{\frac{1}{2}}$$

simplifying:

$$k = \frac{0.693}{t_{\frac{1}{2}}}$$

therefore:

$$t_{\frac{1}{2}} = \frac{0.693}{k} \qquad \text{(Eq. 5)}$$

It should be obvious that the above considerations, while of some value for purely practical purposes, give no indication of the nature of the inactivating event in viral populations exposed to heat. In order to define the molecular basis of virus thermal decay, it is necessary to have some knowledge of the quantitative differences in the specific reaction rates of a virus denatured at more than one temperature. If at least two velocity constants are known, it becomes possible to evaluate the energy of activation of the process. This relationship is given by the following, which is a modification of the Arrhenius equation:

$$\log \frac{k_2}{k_1} = \frac{E}{2.303R} \cdot \frac{T_2 - T_1}{T_1 T_2} \qquad \text{(Eq. 6)}$$

where k_1 and k_2 are velocity constants for the inactivation carried out at the two temperatures (absolute), T_1 and T_2; R is the gas constant (1.987 cal degrees^{-1} mole^{-1}); and E is the energy of activation of the process, usually expressed in calories or kilocalories. By comparing the energy of activation computed for a given virus or viral component with that known to characterize nucleic acids and proteins, it is possible, at least in theory, to identify the nature of the inactivating event or events (16).

In most studies concerned with the overt effect of heat upon viruses, infectivity has been selected (occasionally by choice but more frequently by necessity) as the measured character. Infrequently one finds in the literature reference to a "thermal inactivation point" (T.I.P.) for a given virus. The T.I.P., though poorly defined, is usually taken to mean the temperature at which the infectivity titer of a suspension of the virus is found to decrease rapidly. More typically, data from thermal inactivation studies are expressed either as the net loss or decrease of infectivity of a preparation as a function of time of exposure to a particular temperature. Such kinetic measurements are obviously more quantitative in nature and are therefore of greater value than T.I.P. determinations.

Price (17) has reviewed the early studies of the thermal decay of viruses. In 1886, Mayer, who first demonstrated the transmissibility of tobacco mosaic virus (TMV), observed that the infectivity of sap taken from diseased plants was destroyed by heating for several hours at 80 C. Iwanowski reported in 1893 that sap infectivity was completely lost when

preparations were heated to near boiling, and his observations were confirmed shortly thereafter by Beijerinck, who stated that infectivity was lost when the sap was boiled or kept even briefly at 90 C. He also noted that diluting the infectious sap with water (1:10) markedly decreased the thermostability of TMV.

The first kinetic study of TMV inactivation at elevated temperatures was carried out by Price (18), who confirmed that dilution of infectious sap with either water or normal sap increased the rate of decay of viral preparations. Price also found that when the natural logarithm of the infective titer of TMV suspensions was plotted against the time of heating at 69.8 C, the result is a straight line function, at least over a concentration range of four logarithm units, indicating that under these conditions TMV was inactivated in accordance with first-order kinetics. The energy of activation for the process was found to be high; the E values obtained fell within the range of 55,300-195,000 cal/mole.

Boyd and Eberl (19) reviewed the kinetic data of Price and that of other investigators who had reported similarly high heats of activation for the thermal decay of a variety of other plant viruses. In view of the large amount of energy required to inactivate these agents, Boyd and Eberl postulated that denaturation involves a series of chain reactions, the first requiring the simultaneous breaking of 25-30 bonds of a weak type, such as hydrogen bonds. The succeeding steps in the chain would be essentially spontaneous, requiring little if any net energy. Thus the denaturation process is compared with the tearing of an oriented

film such as cellophane; the force required to initiate the tear being very large, while that required to continue it would be comparatively small.

Ginoza has more recently investigated the thermal decay kinetics of purified TMV ribonucleic acid (RNA) (20). RNA preparations at neutral pH in 0.1M phosphate buffer heated at temperatures between 55 and 65 C demonstrate an energy of activation that is considerably lower than that computed for intact TMV. While the inactivation rate is found to be linear over a concentration range of two logarithm units (at 55 and 65 C), the energy of activation is only about 19,000 cal/mole. Thus the mechanism of heat decay of TMV-RNA appears to be quite distinct from that of the intact virus. Ginoza postulated that the viral nucleic acid in such preparations is inactivated by a single random hydrolytic cleavage of a phosphodiester bond, thus breaking the polynucleotide chain, rather than by the rupture of a series of weak bonds. On the basis of such kinetic evidence, Ginoza predicted the single-stranded nature of the nucleic acid of TMV, an hypothesis that has since been verified (21).

In almost all studies directed at the thermal decay of plant viruses, the denaturation of the particular agent has been shown to follow typical first-order kinetics. An exception is a report by Babos and Kassanis (22), who subjected preparations of tobacco necrosis virus (TNV) to temperatures varying from less than 40 C to as high as 90 C. At the higher temperatures (65-90 C) the usual single-component decay kinetics were observed. However at lower temperatures TNV was found to

be inactivated in a manner suggesting the presence of a second, slower-decaying component. This was indicated by a break in the linearity of the decay plot after several minutes of heating. The slow component was itself linear (thus a semi-logarithmic function), having a slope much less than that characteristic for the rapid component. At temperatures decreasing from 65 C the slow component became increasingly evident, until at 40 degrees and below it was the only component remaining. Babos and Kassanis also inactivated purified TNV-RNA and found kinetics identical to that of the intact virus preparations. They concluded that TNV is inactivated as a result of denaturation of the nucleic acid moiety alone, and that the protein shell of the virus offers little, if any, protection to the enclosed nucleic acid.

Two-component kinetics have also been noted for the thermal degradation of E. coli phage T5. In 1959 Patch (23) found that when T5 suspensions are heated at 65.4 C, the rate of decay is inconsistent with that predicted by adherence to first-order kinetics alone. Patch incorrectly interpreted this deviation from semi-logarithmic linearity as evidence for the curvilinear nature of the decay rate. His data in fact are more accurately interpreted when plotted as a two-component system, each component retaining the linear nature of the first-order reaction.

Fiers and Sinsheimer (24) subjected preparations of isolated deoxyribonucleic acid (DNA) from phage ϕX174 to very high temperatures in the hope of revealing some insight into the structure of the viral nucleic acid. At 98 C (pH 9.0) the phage DNA is inactivated according

to typical first-order kinetics. Fiers and Sinsheimer postulated that inactivation is probably a consequence of depurination rather than chain scission.

Using the T5 system Hershey, Goldberg, Burgi and Ingraham produced DNA breakage by stirring and denaturation by exposure to elevated temperatures (25). It was found that while high solute concentration protected the nucleic acid from denaturation, it had little effect upon the rate of chain breakage, even at high temperatures. They therefore concluded that chain breakage and denaturation are not synonymous in this virus; rather they are independent processes in that they show no particular tendency to occur simultaneously in a single molecule, nor does one predispose to the other.

While investigating the kinetics of denaturation of TMV-RNA, Ginoza et al. (21) examined at the same time and in a similar fashion the decay rates of the single-stranded nucleic acids of two other viruses, the phages R-17 and ϕX174. Their results in general confirmed the hypothesis of Fiers and Sinsheimer (24) concerning the identity of the denaturation event in ϕX174 DNA; i.e., a single depurination inactivates the molecule. RNA from R-17 phage was found to undergo, in the presence of heat, a chain depolymerization, presumably resulting (as in the case of TMV-RNA) from cleavage of a single phosphodiester bond. In both cases only first-order kinetics were observed.

From the above discussion it appears evident that the nature of the denaturation event in plant and bacterial viruses exposed to heat is dependent upon two factors, the presence or absence of capsid protein,

and the type of nucleic acid characteristic of the virus. In preparations containing only viral nucleic acid, the decay kinetics are those of a first-order reaction, with no second or slower component in evidence. The exception to this, as pointed out earlier, is the report of Babos and Kassanis (22). The implication is that the presence of a second component in thermal inactivation plots of intact viruses is due to the effect of heat upon another constituent of the virus which is altered in some fashion so as to render the entire particle non-infectious. A most logical candidate is the viral protein capsid. The type of nucleic acid apparently determines the nature of the inactivating event in that DNA is denatured at high temperatures by depurination, RNA by chain scission.

Certain animal viruses have been well studied with regard to their response to elevated temperature. Vaccinia, poliomyelitis and foot and mouth disease viruses have received particular attention. The earliest thorough kinetic studies were those of Kaplan (26), who examined the heat decay of vaccinia virus in a dilute phosphate buffer, McIlvaine buffer (0.004M PO_4^{---}, pH 7.2). Two types of virus were examined, a Lister Institute vaccine strain adapted to the chick embryo chorioallantoic membrane and a "heat resistant" variant of the same strain which had been observed to survive 60 C for several minutes. The kinetics of decay were found to be identical for both types of virus, consisting of two components distinguishable by order of occurrence and slopes. The "fast" or "high temperature" component, which occurred first, was found to be a first-order exponential function whose slope

and velocity constant were dependent upon temperature. The energy of activation computed from reaction rates at differing temperatures was found to decrease as the absolute temperature increased. The "slow" or "low temperature" component, which becomes evident only after several minutes of heating at 50-60 C, was unique in several ways. Though a linear function, its slope was independent of the temperature, at least over the range tested. Thus the specific reaction rate and energy of activation of this component remained constant. Kaplan observed that the extrapolated y-intercept of the slow component ascended the ordinate (increasing infectivity) with decreasing temperature. At the lowest temperature tested, 50 C, it constituted the principal portion of the decay plot. Unfortunately, no lower temperatures were tested, for the obvious implication is that there exists a temperature at which only the slow component functions, its y-intercept having a value of 100% or zero loss of infectivity at zero time. Kaplan interpreted his data as an indication that there are two processes involved in the thermal inactivation of vaccinia virus, both of which operate simultaneously. One of these processes is wholly temperature-dependent with a relatively high energy of activation or Q_{10}; the other is relatively temperature-independent within certain limits, proceeding at the same rate at T as at $T + 10$.

Inactivation of foot-and-mouth disease virus (FMDV), a heat-labile member of the picornavirus group, has been studied in some detail by Bachrach, Breese, Callis, Hess and Patty (27). These investigators examined the effect of pH and a wide variety of temperatures on FMDV,

type A, strain 119 (tissue-culture derived). They found that the virus is stable only within a very narrow range of pH, from 7.0 to 7.5. Outside these limits decay proceeds very rapidly even in refrigerated preparations. Thermal inactivation experiments were performed on virus preparations suspended in veronal-acetate buffer, pH 7.5, at temperatures from 4-61 C. Ninety per cent of the viral infectivity was lost in 18 weeks at 4 C, 11 days at 20 C, 21 hr at 37 C, 7 hr at 43 C, 1 hr at 49 C, 2 min at 55 C and 30 sec at 61 C. At temperatures of 43 C and below, only one component was observed in the decay plot, while at temperatures of 49 C or higher two components were readily apparent. An Arrhenius plot of the kinetic data indicates that two processes are involved in the thermal decay of FMDV. Activation energy computed for the inactivation of the virus below 43 C was 27,200 cal/mole, while above 43 C the activation energy was found to be 120,600 cal/mole. From a consideration of the earlier work on plant viruses, the authors conclude that at very high temperatures the virus is inactivated by denaturation of the protein moiety, while at lower temperatures decay is the result of some unspecified alteration of the viral nucleic acid. In a later paper (28), Bachrach verified to his own satisfaction this hypothesis. He heated FMDV in the presence of sodium dodecylsulfate to temperatures as high as 85 C. The role of the detergent was to inactivate any environmental ribonuclease (RNase) which might be present. Under these conditions RNA is quantitatively released in a fully infectious form from the virion, presumably by rupture of the viral capsid.

Brown, Cartwright and Stewart (29) have also studied the inactivation of FMDV by a number of means including exposure to heat. They found that at low temperatures (25 and 37 C), heating destroys infectivity without causing any noticeable change in the serological specificity of the protein. Heating at 56 C causes release of infectious RNA and complete loss of serological reactivity. This study seems to confirm the hypothesis of Bachrach.

Dimopoullos (30) has been critical of attempts to define the physicochemical characteristics of FMDV by examination of tissue-culture adapted strains alone. He has tested suspensions of infected bovine tongue epithelium and found evidence of continued infectivity and complement-fixing activity after much longer periods of heating than have been reported for tissue-culture adapted strains. In one experiment, for example, complement-fixing ability was clearly present after 24 hr at 56 C and 30 min at 70 C. It is unfortunate that Dimopoullos has relatively little quantitative data describing the relationship between immunological properties and infectivity in his system. At any rate, the use of crude tissue suspensions of the virus might profoundly effect the decay of both serological specificity and infectivity, perhaps sufficiently to cause the differences noted.

The mechanism of heat inactivation of poliovirus has been particularly well studied, probably as a result of the intense interest shown in poliovirus vaccines in the last fifteen years. In general, the kinetics of poliovirus inactivation at elevated temperatures are similar to those found for FMDV and other picornaviruses; i.e., at temperatures

greater than 45 C two components are observed, while at temperatures less than 45 C only one component is evident (31).

Norman and Veomett (32) studied the inactivation of poliovirus and purified preparations of viral RNA at temperatures from 40 C to 75 C. They found that at temperatures higher than 60 C the RNA preparations were more stable than whole virus suspensions. Their conclusion was that under these conditions poliovirus is inactivated by alterations in the protein coat sufficient to render the virion noninfectious, rather than by denaturation of the nucleic acid. The heat of activation for the decay of RNA preparations was computed as 31,000 cal/mole, and the entropy of activation was 21 cal/mole/degree. The positive entropy value is at variance with the negative value (-19 cal/mole/degree) reported for TMV-RNA by Ginoza (20). Norman and Veomett therefore postulated that either the structure of the nucleic acids of the two viruses are decidedly different or denaturation of poliovirus RNA occurs in a radically different way. They consider that the former possibility is the most likely.

Gordon, Huff and Holland have examined the nucleic acids of both TMV and poliovirus in an effort to resolve this question (33). Their results conflicted with those of Norman and Veomett. Testing the nucleic acids at temperatures of 65, 80 and 100 C under the same conditions of ionic strength and pH, they found inactivation kinetics that were almost identical. They conclude that there is no reason to postulate an intrinsic difference in the chemical structures of these two RNA molecules.

Papaevangelou and Youngner (34) have investigated the kinetics of the heat decay of two Type I (Brunhilde) poliovirus strains and their respective nucleic acid fractions. The strains differ principally in their response to heat; one strain demonstrates typical poliovirus lability, while the other (presumably a mutant) is markedly heat-resistant. Two types of experiments were performed. Decay of a purified RNA preparation was compared with that of the corresponding whole virus, and the inactivation kinetics of the labile virus and the stable mutant were compared, as were the respective nucleic acid fractions. Papaevangelou and Youngner found that RNA from the labile virus was inactivated more slowly than the intact virus, while RNA from the stable strain was inactivated at the same rate as the whole virus. They also found that RNA from the labile strain was much more heat-sensitive than RNA from the stable mutant. They conclude that in the case of the labile virus the primary event in heat decay is some unspecified alteration in the protein of the capsid which leads to loss of infectivity of the virion, as has been previously suggested for FMDV (27,28). No such conclusion can be drawn for the stable mutant, as the decay rates for both RNA and the intact virus are the same. The implication is that inactivation in this case is due to denaturation of the nucleic acid component, but this can only be supposition. The stability of the variant does appear, however, to result from a change (mutation) in the genome, as the infectious RNA fractions are clearly distinguishable on this basis.

Pohjanpelto (35) has recently reported her attempts to repeat the

experiments of Papaevangelou and Youngner. Using the same virus strains and conditions, she was unable to demonstrate any difference in the decay rates of the RNA fractions of the two strains. The greater sensitivity of the whole virus suspensions to heat was, however, noted. She concluded that poliovirus, as well as other picornaviruses, is inactivated at high temperatures by denaturation of the protein coat. Pohjanpelto postulated no mechanism for this event.

A concerted effort directed toward determining the precise molecular nature of poliovirus inactivation has been made by several groups of workers. There is good evidence that heating suspensions of poliovirus causes distinct and reproducible changes in the serological character of the virus (36,37,38). Watanabe, Watanabe, Katagiri and Hinuma have shown that in such preparations there occurs dissociation of the viral nucleic acid from the protein capsid (39). Recently this same group attempted to correlate these two events by following the dissociation of viral components (of known antigenic character) with the aid of the electron microscope (40). They found that when Type 1 Mahoney strain poliovirus is inactivated at 55 C, virions with "H" antigenicity were converted to empty capsids with "N" specificity. The kinetics of RNA release and antigenic conversion were studied and found to be identical. They conclude that the dissociation of RNA from the capsid causes a configurational change in the viral protein sufficient to alter the serological specificity of the capsid material. The converse is, of course, equally possible; i.e., the alteration of the capsid protein (which is evidenced by the antigenic change noted) is the primary event

which triggers release of the RNA from the particle. Thus far it has been impossible to resolve these possibilities. Once denuded of its protective protein coat, the viral RNA is susceptible not only to heat but also to the action of even minute amounts of environmental RNase. Thus the kinetics observed in the heating of poliovirus suspensions may be artifacts of a double event, the splitting of the capsid, which is a result of heat, and the inactivation of the free nucleic acid, which may be due either to heat or to enzymatic activity. There is every reason to believe that many other viruses, particularly picornaviruses, are inactivated in a similar fashion.

The nature of the thermal decay process as regards the arthropod-borne group of viruses (arboviruses) is poorly understood, although considerable study has been directed at these agents. The extent of information available for most members is typified by a recent report by Parodi, Coto, Boxaca, Lajmonovich and Gonzalez (41). These investigators studied the response of Junin virus to a variety of temperatures in the presence of isotonic phosphate buffer and 2% normal rabbit serum. They found little or no loss of infectivity after 26 hr at 25 C and 6 hr at 37 C. Inactivation was found to be essentially complete (99.9% loss of infectivity) after 192 hr at 4 C, 72 hr at 25 C, 26 hr at 37 C and 10 min at 56 C.

The most significant advances in elucidating the mechanism of the inactivation process for this group of viruses have resulted from parallel whole virus-infectious nucleic acid studies. While relatively few arboviruses have been studied by this method, there appears to be a

degree of consistency among those so investigated. Ada and Anderson (42) compared the titer of infectious RNA isolated from unpurified preparations of Murray Valley encephalitis virus with intact virus, following heat treatment at 55 C for one hour. They found that the infectivity titers of the pre-heated RNA were considerably higher than the corresponding virus suspensions pre-heated in the same way. Similar findings have been reported by Nakamura for mouse encephalomyelitis virus (43).

Nakamura and Ueno (44) have performed a similar comparative study as part of a more intensive investigation of Japanese B encephalitis virus. The thermal stability of intact virus and purified infectious RNA were compared under a variety of conditions of pH and ionic environment. The decay of the crude virus suspension (a 10% infected mouse brain homogenate in distilled water) at 50 C was found to parallel that of the purified infectious RNA if the pH was maintained near neutrality. Both preparations were inactivated in a two-component fashion. The rate of decay, however, varied considerably with changes in salt (NaCl) concentration and pH. At low salt concentrations intact virus was most stable at alkaline pH, while liberated infectious RNA was most stable at pH 4-5. Very high salt concentrations stabilized isolated nucleic acid while enhancing the degradation of intact virus.

Mika, Officer and Brown (45) have extensively investigated the response of two arboviruses, Eastern and Venezuelan equine encephalitis (EEE and VEE), to high temperatures, ultraviolet light and nitrous acid. Both viruses demonstrated two-component decay kinetics when heated at

50 C. In the preparations containing EEE, the slope of the first component was the steepest; the reverse (i.e., slow first component) was found for VEE. Some 99% of the total infectivity of EEE virus suspensions was lost in one hour at 50 C. Three hours were required for the same degree of inactivation with the Venezuelan agent. Both extracted and recoverable RNA (nucleic acid isolated before and after heating) were more resistant to heat than either corresponding intact virus. The authors concluded that the early (first component) inactivation is probably a surface or surface-related event which "kills" the virus by destroying cell-attachment sites. The later phase (second component) is probably due to nucleic acid denaturation. This hypothesis, as yet untested, might well explain the findings of other investigators regarding arboviral heat inactivation.

D. Agents Modifying the Response of Viruses to Heat

The sensitivity to heat of a virus particle is strongly influenced by its environment. Crude or concentrated virus suspensions, containing large amounts of host cell debris, proteinaceous diluent (e.g., serum or its derivatives), noninfectious whole virus and viral components, are relatively more stable at high temperatures than either dilute unpurified or purified preparations. The reasons for the greater stability of such crude or concentrated suspensions are incompletely understood, although at least two factors are known to play significant roles: 1) the nonspecific protein-sparing effect of many large molecules; and 2) specific stabilization of viral infectivity by small molecules. The subject of

the following discussion is the latter specific small molecule effect.

The earliest demonstration of specific virus stabilization was reported by Adams and Lark in 1950 (46). These investigators noted that purified preparations of wild-type phage T5 diluted in distilled water were extremely labile, rapidly losing infectivity at temperatures as low as 37 C. The addition of very small amounts of divalent cations was found to significantly stabilize such suspensions. Calcium and magnesium salts (chlorides) were found to be particularly efficient in this regard. Dilution in as little as 10^{-3} M $CaCl_2$ or 10^{-2} M $MgCl_2$ fully stabilized (i.e., no detectable loss) T5 phage infectivity at 37 C for prolonged periods of time.

Hershey, Goldberg, Burgi and Ingraham (25) found in the same phage system that the dilution of the viral DNA in 2.6 M NaCl markedly protected the nucleic acid to heat-induced denaturation while having no effect upon DNA breakage resulting from rapid stirring.

Tucker (47) has attempted to define the role of Mg^{++} in bacteriophage ϕR (a small coliphage containing single-stranded DNA) in terms of one or more events occurring in the replicative cycle. In one-step growth experiments with this phage he noted that the yield of phage per infected cell increased with increasing Mg^{++} concentration. The increase "plateaued" at 10^{-2} M $MgCl_2$; higher concentrations had no further effect. The function of the cation did not appear to be simply that of a lysis cofactor since the titer of intracellular phage at any time was also dependent upon ion concentration. Phage development could

be appreciably promoted by addition of Mg^{++} even when the ion was added late in the replication cycle. The result was not affected by the simultaneous addition of chloramphenicol or a variety of respiratory poisons. The precise role of Mg^{++} in this phage/host system remains unclear.

In contrast to the cation effect, monovalent anions have been found to markedly enhance the thermal decay of T2 DNA. Hamaguchi and Geiduschek (48) have examined the effect of a wide variety of simple salts on the stability of purified aqueous nucleic acid suspensions. The negative nature of the effect was noted by observing a decrease in the 50% thermal melting point (T_m) of the DNA. Salts containing $COOH^-$, Cl^- and Br^- decreased the T_m from 43 to 3.2 C at 0.1 M concentrations. Salts containing the anions CH_3COO^-, I^-, CF_3COO^-, CNS^- and CCl_3COO^- had a similar, though less drastic effect.

There is considerable evidence that several plant viruses are markedly stabilized by the addition of various cations. Magnesium ion (as the chloride salt) has been clearly shown, for example, to protect potato yellow dwarf virus (49), alfalfa mosaic virus, cucumber mosaic virus, tobacco mosaic virus, tobacco necrosis virus, tobacco rattle virus (50) and brome mosaic virus (51) when present at 0.01-0.1 M concentrations.

Staehelin has examined the degree of structuring of tobacco mosaic virus RNA under a variety of conditions (52). The degree of structural rigidity is reflected, according to Staehelin, by optical rotation,

spectrophotometric and specific sedimentation values. When, for example, viral RNA is diluted in distilled water, the preparations absorb ultraviolet light to an extent far greater than preparations diluted in 0.1-1.0 M $MgCl_2$. Staehelin postulates that the hyperchromicity displayed by TMV-RNA in the presence of Mg^{++} results from the transition from a random coil configuration (in distilled water) to a more structured form. Such a change could conceivably result, for example, from the formation of purine-pyrimidine hydrogen bonds (as in DNA); thus double or even triple-stranded helices might result from the effect of the cation on the negative charges of phosphate groups. The net effect of such salts would be a more highly structured or organized form of the nucleic acid, which presumably would be less susceptible to the disruptive effects of heat. Boedtker (53) has performed essentially identical experiments, though at a much lower total salt level. His conclusions largely parallel those of Staehelin.

Huff, Sastry, Gordon and Wacker (54) have reported the same sort of experiment as that discussed above with, however, conclusions that in part contradict those of Boedtker and Staehelin. Huff and his coworkers found that 10^{-3} M Ni^{++} and Mg^{++} do in fact stabilize the secondary structure of TMV-RNA, presumably by the mechanism postulated by Staehelin. However, at 65 C these same ions, as well as Mn^{++}, Ca^{++}, Zn^{++} and Co^{++} (all as chlorides), were found to enhance the thermal decay rate of the nucleic acid, rather than exerting a stabilizing influence. Chelating agents (e.g., 0.1% EDTA) were found to moderate

the destructive effect of the cations, though not to reverse it. There is, it would appear, a paradox of sorts at hand. Added metal ions, while stabilizing TMV-RNA secondary structure, cause a more rapid loss of biological activity in heated suspensions. Huff, et al. (55) postulated that the loss of infectivity is the result of metal ion-catalyzed hydrolysis of phosphodiester bonds, while secondary structure stabilization probably results from the formation of internucleotide (phosphate-phosphate) metal bonds. Even after a hydrolytic break in the polynucleotide chain had destroyed all biological activity (infectivity) the same molecules might retain secondary structure stability as evidenced by ultraviolet absorption hypochromicity. The paradox might therefore be only apparent.

The first report of specific small ion stabilization of an animal virus resulted from the early attempts of Kaplan and Micklem to prepare a vaccine strain of vaccinia virus in quantity from tissue cultures. These investigators noted that the infectivity of vaccinia preparations was considerably influenced by the concentration of phosphate ion in the diluting buffer. After ten days at 22 C the infectivity titer of virus stored in McIlvaine phosphate-citrate buffer, 0.004 M in PO_4^{---} ion was 1.6×10^7 egg infectious units (EIU) per ml. Increasing the phosphate ion concentration to 0.1 M resulted in a fivefold increase in titer (7.9×10^7 EIU/ml) after ten days at the same temperature. In a later paper Kaplan (56) evaluated the response of vaccinia virus to a variety of metal cations. He found that monovalent ions (Na^+, K^+) were

more effective in stabilizing the virus than were the divalent ions Mg^{++} and Ca^{++}. Heavy metal ions (Cu^{+++}, Co^{+++} and Fe^{+++}) were noted to cause rapid and complete loss of infectivity. Optimum stabilization, however, resulted when Na^{+} and Mg^{++} were used jointly, both at 0.1 M concentrations. Under these conditions vaccinia preparations were virtually stable. Kaplan postulated that the protective effect of small metal ions was due to the formation of metalloprotein complexes less subject to the effects of heat.

Wallis, Yang and Melnick (57) have also investigated the response of vaccinia virus to high concentrations of metal cations of various sizes. They also compared the reaction of vaccinia to that of two smaller DNA viruses, herpes simplex and adenovirus, types 4 and 7. Sodium ion at 2 M concentration was found to stabilize vaccinia virus more effectively at 4, 25 and 37 C than 50% glycerin. The heavier cations, Mg^{++} and Ca^{++}, exerted a clearly detrimental effect on this virus, even when compared as diluents with distilled water. Strontium, barium, manganese, zinc, copper and cobalt ions also quickly inactivated the virus. Herpes simplex and the adenovirus strains were stabilized only by 2 M Na^{+} at temperatures as high as 50 C; all other ions tested enhanced inactivation of these agents.

Rapp, Butel and Wallis (58) have reported the rapid inactivation of measles virus and other myxoviruses by $MgCl_2$. The sulfate anion, in contrast, appears to stabilize this group of viruses, even when present as the magnesium salt. All sulfate salts tested by these workers

($MgSO_4$, Na_2SO_4 and K_2SO_4) clearly stabilized measles virus at 45-50 C. A direct concentration response was noted, with maximum protection occurring at molar concentration levels. Chloride salts of the same cations had either no appreciable effect or, in the case of $MgCl_2$, a clearly deleterious one.

Among the animal viruses the most thoroughly investigated in this regard have been the enteroviruses. The intensive study directed at this group of agents has resulted, in part, from the need to develop means of prolonging the storage life and eliminating adventitious viral contaminants of the various poliovirus vaccines. Wallis and Melnick reported in 1961 (59,60) the simultaneous stabilization of vaccine-type poliovirus strains and the rapid inactivation of the latent simian virus 40 (SV40) by treatment of viral suspensions at 50 C with molar $MgCl_2$. Under these conditions the poliovirus strains were apparently stable, while the DNA-containing SV40 underwent rapid and complete decomposition. Wallis and Melnick postulated that the difference in susceptibility of these two viruses to heat in the presence of the salt was a function of the nucleic acid characteristic for the virus.

In a later paper (61) Wallis and Melnick reported the results of heat inactivation experiments in the presence of 1 M Mg^{++} and Ca^{++}, 2 M Na^{+} and distilled water with 35 enterovirus serotypes, three reovirus strains, three adenovirus types, two papova viruses, herpes simplex, three myxoviruses (influenza, mumps and parainfluenza), an arbovirus (Sindbis) and a poxvirus (vaccinia). Among the viruses tested

only the enteroviruses and reoviruses were stabilized by high concentrations of the indicated ions. All other viruses were more rapidly inactivated at 50 C in the presence of the cations than in distilled water. Wallis and Melnick noted that among the spectrum of viruses tested in this system only those with demonstrated cubic symmetry, an RNA core and absence of essential lipid (lipid required for infectivity) were stabilized by magnesium ion. DNA viruses with the same type of symmetry (cubic), such as the adenoviruses, papovaviruses, and the herpesviruses, RNA viruses with helical symmetry (myxoviruses) and the two viruses with undetermined symmetry (Sindbis and vaccinia) were rendered more thermolabile by the cation. Wallis and Melnick therefore predicted that all small RNA viruses possessing cubic or icosahedral symmetry, lacking any essential lipid, would, if tested in this system, demonstrate increased heat stability in the presence of $\underline{M}$ $MgCl_2$. Subsequent efforts by several workers (62,63,64) have verified this hypothesis.

The impact of this discovery can perhaps best be measured by the variety and number of practical advances that have resulted from application of the cation-stabilization theory to specific problems. The shelf or storage life of live attenuated poliovirus vaccines has been lengthened from two or three days at 23 C to several weeks. It is no longer necessary to ship the vaccine in the frozen state, nor to refrigerate it in the field (65,66). Dilution of viral antigens in molar Mg^{++} simultaneously stabilizes the desired antigen and inactivates

most contaminant agents (64). The preparation of killed virus vaccines can be simplified by use of the cation either to enhance the antigenic potency of formalinized vaccines (67) or as the primary inactivating agent (68). The aluminum cation (Al^{+++}) has been found to selectively stabilize attenuated but not neurovirulent poliovirus strains; attenuated strains can therefore be "cleansed" of virulent variants from viral populations known or suspected of being heterogeneous (69,70).

Relatively little is known of the response of most arboviruses to high concentrations of small molecules. Osterrieth (71) has reported the stabilization of Semliki Forest virus by 0.14 M sucrose, 0.1% gelatin and 0.54 M glycerol. The degree of protection observed with these diluents was, however, only partial; approximately half of the original infectivity was lost in one hour at 37 C in their presence. The reducing agents sodium hyposulfite and sodium thioglycollate exerted a marked destructive effect upon viral suspensions even at very low (0.005 M) concentrations.

Mayerova (72) investigated the survival of Tahyna virus (an arbovirus of the California group) in molar concentrations of calcium and magnesium chlorides. Viral suspensions were diluted 1:10 in the concentrated salt solutions and heated at 37 or 50 C for 30-60 min. The arbovirus was found to undergo much more rapid decay in the presence of either cation than in phosphate buffer alone. At both 37 and 50 C inactivation was approximately one hundred-fold greater in the salt solutions than in the isotonic buffer.

Mayer and Slavik (73) have similarly examined the thermal stability of both purified (via density gradient) and unpurified European tick-borne encephalitis virus (a group B arbovirus) in the presence of a variety of salts of monovalent metallic cations. The stability of virus heated at 50 C for 10 min was found to increase appreciably, according to the authors, when virus was diluted in 0.2-1.0 M cation solutions. Both unpurified and purified virus were protected to approximately the same extent. It is unfortunate that Mayer and Slavik did not mention in their report the particular salts they tested, nor are the decay data provided.

Nakamura and Ueno (44) discovered a most interesting cation effect while investigating the basic physical properties of Japanese B encephalitis virus and its associated ribonucleic acid. The authors carried out thermal inactivation experiments on both whole virus and purified nucleic acid isolated from the virus. They found that while high concentrations of NaCl enhanced the inactivation of intact virus, it protected the nucleic acid preparations. In both cases there was a very clear "dose-response" effect; the types of response, however, were precisely opposite. In a more recent publication (74) this phenomenon was explored in somewhat greater detail with, unfortunately, equally confusing results. To summarize: intact virus was protected at 50 C by prior dilution in 1 M Na_2SO_4 or 1.5 M $MgSO_4$; accelerated inactivation resulted from exposure to 0.1-1.0 M NaCl, 0.1 M Na_2SO_4 and 0.15 M $MgSO_4$. Isolated and purified viral RNA was stabilized by 1.0 M NaCl and to a

lesser extent by 1.0 M Na_2SO_4; dilution in 0.1 M NaCl, 0.1 M Na_2SO_4, 0.15 M $MgSO_4$ and 1.5 M $MgSO_4$ resulted in very rapid decay.

Barnes has examined the thermal inactivation kinetics of Sindbis virus in the presence and absence of several sulfates.[1] He, too, found a degree of variation in the response of the virus to the sulfate anion; the net effect of the salt on the virus was found to be a function not only of the sulfate moiety but also the cation. In virus preparations heated (47-56 C) in the absence of added salt the virus became more susceptible to the action of RNase as the time of heating increased. The implication is that the primary inactivating mechanism of increased temperature with this virus is an opening of the protective protein capsid with subsequent denaturation of the viral nucleic acid by environmental factors. In the presence of molar quantities of any of several sulfate salts, exposure to high temperatures evoked little change in the susceptibility of the virus to added enzyme. Barnes feels that the protective effect of the sulfate anion is due to the ability of the ion to prevent this rupture.

E. Pertinent Studies on Colorado Tick Fever Virus (CTFV)

Colorado tick fever (CTF) in human beings is a usually mild, biphasic, granulocytopenic, febrile illness of viral etiology. Encephalitis and other complications occur in a minority of cases. The disease

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[1]Barnes, R. Personal Communication, 1967.

is endemic in most of the mountainous western United States. Actively acquired immunity is known to be of long duration. The tick Dermacentor venustus (syn. andersoni) is the principal vector; small rodents are believed to function as a natural reservoir (75,76).

Florio, Stewart and Mugrage first reported the isolation of the infective agent (77). It was recovered from moribund hamsters following injection of serum from a patient in the acute phase of the disease. In a later paper, Florio, Stewart and Mugrage (78) identified the agent as a small virus capable of passing through a membrane having an average pore diameter of 24mμ.

The original Florio strain of CTFV was successfully adapted to adult dilute brown agouti (DBA) mice, adult white mice and embryonated eggs, in which it proliferated abundantly in the central nervous system of the embryo (79). Suckling mice were found to be particularly susceptible to all strains of the virus (80,81) and are still the principal means of virus isolation from blood specimens and infected ticks.

Pickens and Luoto demonstrated proliferation of CTFV in a stable tissue culture cell line (KB) of malignant human epidermal origin (82). Gerloff and Eklund developed a tissue culture neutralization test for the demonstration of either the virus or specific antibody (83). Later Thomas and Eklund (84) devised a diagnostic complement fixation test of considerable epidemiologic value based upon a modification of an earlier method (85).

Gardner (86) has examined in detail the tissue culture sensitivity

of three mouse brain strains of the virus. Two of these strains, Lederle-1160 and Florio-2, failed to propagate in primary chick embryo, mouse embryo, hamster embryo, chick kidney, mouse kidney, monkey kidney and hamster kidney cell cultures. Equally resistant were the established cell lines he examined (parent and cloned HeLa, Chang's Human Liver, KB and KB-23). Only the Detroit-6 cell line was susceptible to mouse brain preparations of these two strains. A third CTFV strain, GS-20 (83), was found to propagate in both KB and KB-23, as well as Detroit-6, cell cultures. Gardner was unable to demonstrate the presence of a hemagglutinin for the virus, nor were attempts to detect progeny virus in infected cell cultures via specific immunofluorescence successful.

Trent and Scott (87) have compared the sensitivity of several cell lines to mouse brain and cell-adapted CTFV, Florio strain. These investigators found that this strain infects and replicates in the L, FL and KB lines and in primary chick embryo fibroblasts. The L, FL, and chick embryo cells were as sensitive to infection as were suckling or weanling mice inoculated intracerebrally with the agent. Both the HeLa cell line and primary cotton rat kidney cultures were totally resistant. In infected L-cell cultures, 90% of the input virus was adsorbed to the cells in the first 30 min of incubation. The viral latent period was found to last 10-12 hr.

In a later paper (88) Trent and Scott reported studies on the physical and chemical properties of the Florio strain. Heat inactivation

kinetics for preparations of the virus grown in L-cells indicated that more than one component was involved in the inactivation process at temperatures from 25 to 56 C. The energy of activation computed for thermal inactivation of the virus was 17,289 cal/mole. The half-life of the L-cell-grown virus was 21 min at 37 C and 72 min at 25 C. The optimum pH range for maintenance of viral infectivity was found to be 7.5-7.8. A variety of diluents (50% calf serum, 20% glucose, 20% glycerol, 10% bovine serum albumin, 20 mM glutamine and 2% gelatin) were noted to effectively protect virus infectivity during repeated freeze-thaw manipulation. Trent and Scott also found that CTFV replication was insensitive to 5-fluoro-2'-deoxyuridine and 5-bromo-2'-deoxyuridine; actinomycin D failed to inhibit viral replication when added to host cells at the time of infection. The implication is that Colorado tick fever virus contains an RNA core (88,89).

A plaque assay method for CTFV in which an embryonic hamster (A-1) tissue cell strain was used as host was described in 1964 by Deig and Watkins (90). The method, although workable, was a fragile one, susceptible to very slight changes in a variety of environmental conditions. Unfortunately, the indicator cell line has since undergone significant and apparently permanent changes of an undetermined nature effecting a decrease in sensitivity to the virus.[1] A similar assay

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[1]Deig, F. E. Personal Communication, 1964.

method incorporating the mouse L-cell as host has been developed by Frazier (91). This plaquing technique has proven to be a sensitive and reliable method for the detection and quantitation of several CTFV strains.

MATERIALS AND METHODS

A. The Virus

The GS-20 strain of CTFV was used throughout this study. This variant was derived by Gerloff and Eklund in 1959 from the Florio strain of CTFV adapted to 21-day-old mice. They obtained the Florio strain in the 37th mouse brain passage from Dr. Herald R. Cox of Lederle Laboratories. Gerloff and Eklund passed the agent twenty times in mice and seven times in embryonated eggs. Following the egg passages, the virus was found to be highly infectious for KB cells. This formed the basis of Gerloff and Eklund's tissue culture neutralization test (83). These investigators noted that peak virus titers with the GS-20 strain were obtained in the brains of 3-day-old mice inoculated intraperitoneally. The brains of animals so infected were found to yield virus titers of approximately $4 \times 10^{5.5}$ fifty per cent cell culture infectious doses ($CCID_{50}$)/g when assayed in KB cells. In the course of their studies Gerloff and Eklund passed the agent four more times in the brains of suckling mice.

Gardner (86) obtained the GS-20 variant from Gerloff in 1961 and passed it six times in mice via the intracerebral route. Three additional mouse brain passages were made by the writer; these passage harvests were used for all the experiments reported herein. The method used for virus passage was essentially that of Gardner. Two- to five-day-old Swiss albino mice were inoculated intracerebrally with 0.01-0.02 ml of a virus preparation containing approximately 10^6 $CCID_{50}$ units.

Virus inoculum was prepared by diluting stock virus 1:10 in 0.85% NaCl containing 10% normal rabbit serum (NRS). The mice were observed daily for signs of encephalitic involvement, and when moribund, they were killed and stored at -70 C. The brains of infected mice were excised, weighed and ground in a sterile mortar and pestle using sterile alundum as a grinding agent. The resulting slurry was diluted in NRS to a final infected tissue concentration of 10% (W/V) and centrifuged at 2,000 rpm for 20 min at 4 C. The supernatant fluid, which contained the virus, was dispensed in sterile ampoules. These were flame sealed and stored at -70 C. The first (M57E7M11) and third (M57E7M13) virus lots prepared by the writer contained no added antibiotics. Penicillin (100 units per ml) and streptomycin (100 µg per ml) were added to the second lot (M57E7M12).

B. Solutions and Media

Accepted procedures for washing and care of all glassware used in cell culture studies and reagent preparation were used (92). The detergent used for all glassware cleaning was 7-X (Linbro Chemical Company, New Haven, Conn.). Double glass-distilled and deionized water (DDW) was used for media and reagent preparation and for all glassware rinsing procedures. Unless otherwise stated, all solutions and media were stored at 4 C.

1. Antibiotics

Antibiotic solutions were used where deemed necessary to prevent the growth of microbial contaminants. Such antibiotics were employed

only at less than toxic concentrations (92).

A stock solution containing a mixture of 20,000 units potassium penicillin G (Charles Pfizer and Company, New York) and 20,000 µg streptomycin sulfate (E. R. Squibb and Sons, New Brunswick, New Jersey) per ml was prepared in sterile DDW and stored at -20 C. One-half ml of this stock solution was added to each 100 ml of medium in order to yield a final concentration of 100 units/ml and 100 µg/ml of penicillin and streptomycin respectively.

Amphotericin B (E. R. Squibb and Sons, New Brunswick, New Jersey) was prepared as a stock solution in sterile DDW; the stock solution contained 12,000 µg/ml. This antibiotic was also stored at -20 C. One-third ml of the stock solution was added to each 100 ml of certain media (final concentration, 40 µg/ml).

Crystalline kanamycin sulfate (donated by Bristol Laboratories, Schenectady, New York) was dissolved in sterile DDW to a concentration of 20,000 µg/ml and stored at -20 C. One-half ml of this stock solution was added to each 100 ml of certain media. The final concentration of kanamycin sulfate, when employed, was 100 µg/ml.

All cell culture media and salt solutions contained both penicillin and streptomycin at the concentrations indicated; amphotericin B and kanamycin were added only to media used for culture of stock cell cultures, never to cells in the presence of virus.

2. Sodium Bicarbonate

A stock solution containing 7.5% $NaHCO_3$ (W/V) in DDW was prepared

as required and sterilized either by filtration through a 0.45 μg (average pore diameter) membrane (Millipore Filter Corporation, Bedford, Mass.) or by autoclaving at 10 lb/in^2 for 10 min.

3. Earle's Balanced Salt Solution (93)

The composition of Earle's balanced salt solution (EBSS) is given in the Appendix. This solution was purchased as a sterile ten-fold concentrate, without $NaHCO_3$, from either Baltimore Biological Company, Baltimore, Maryland (BBL) or from Grand Island Biological Company, Grand Island, New York (GIBCO). For use, 10 ml of the stock solution and 3 ml of 7.5% $NaHCO_3$ were added in order to 87 ml of sterile DDW.

4. Basal Medium Eagle (94,95)

The chemical composition of this medium (BME) is given in the Appendix. Vitamin and amino acid components of this medium were obtained as hundred-fold concentrated solutions from either Microbiological Associates, Inc., Bethesda, Maryland (MBA) or from GIBCO. Single-strength EBME (i.e., BME containing EBSS) was prepared as follows:

Order of Addition	Component
1	83 ml sterile DDW
2	10.0 ml EBSS (10X)
3	1.00 ml BME amino acids (100X)
4	1.00 ml BME vitamins (100X)
5	1.00 ml L-glutamine, 200 mM

6	0.50 ml penicillin/streptomycin mixture (200X)
7	0.50 ml kanamycin (200X)
8	3.00 ml 7.5% $NaHCO_3$
9	0.33 ml amphotericin B (300X)

The L-glutamine and BME vitamin concentrates were stored at -20 C.

5. Hanks' Balanced Salt Solution (96)

Twenty-fold concentrated stock solutions for preparing Hanks' balanced salt solution (HBSS) were made as follows:

Solution A

Components	Amount
NaCl	160.0
KCl	8.0
$MgSO_4 \cdot 7H_2O$	2.0
$MgCl_2 \cdot 6H_2O$	2.0
$CaCl_2$	2.8

The first two components were dissolved in approximately 600 ml of DDW. Two separate solutions containing the magnesium ($MgSO_4$, $MgCl_2$) and calcium ($CaCl_2$) salts were prepared by dissolving the respective salts in approximately 100 ml of DDW each. Each solution was separately autoclaved (10 lb/in^2), cooled, and combined aseptically. Sufficient sterile DDW was then added to bring the final volume to 1 liter.

Solution B

Components	Amount
$Na_2HPO_4 \cdot 7H_2O$	1.8
KH_2PO_4	1.2
dextrose	20.0
0.5% phenol red solution	80.0

These components were dissolved in sufficient DDW to make a total of 1 liter of solution. Phenol red solution was obtained from MBA. Solution B was dispensed into bottles and sterilized by autoclaving at 10 lb/in^2 for 10 min. HBSS is a component of the lactalbumin hydrolysate medium described below.

6. Lactalbumin Hydrolysate Medium (LaH)

This medium was prepared by dissolving 5.0 of lactalbumin hydrolysate, obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio (NBC), in approximately 800 ml of DDW. The volume of this solution was then brought to 900 ml with DDW, and 90-ml aliquots were dispensed in bottles. The medium was sterilized by autoclaving at 10 lb/in^2 for 10 min. Immediately before use 5.0 ml each of the sterile HBSS twenty-fold concentrated solutions A and B were added to each 90-ml volume of LaH. One-half ml of 7.5% $NaHCO_3$ was also added as a buffering agent.

7. Saline A (97)

This buffered saline solution was used for all cell-washing and trypsinizing procedures, as will be described. A ten-fold concentrated stock solution of the reagent was prepared as follows:

Components	Amount
NaCl	80.0
KCl	4.0
dextrose	10.0
0.5% phenol red	40.0

The components were dissolved in sufficient DDW to make 1 liter. The resulting solution was sterilized by filtration and dispensed into bottles in 100-ml aliquots. Saline A was prepared for use by adding 10.0 ml of the stock solution and 0.5 ml of 7.5% $NaHCO_3$ to 88 ml of sterile DDW.

8. Trypsin

Powdered reagent grade trypsin (1-300) was obtained from NBC. A stock solution of the enzyme was prepared by dissolving 0.50 g in 200 ml of Saline A. The resulting solution was sterilized by filtration and stored in 1-ml aliquots at -20 C. This stock solution was diluted either 1:5 or 1:10 (depending on the cell type) in Saline A immediately before use.

9. Normal Sera

Sterile lyophilized calf serum was obtained from BBL and stored at -20 C. It was reconstituted with the proper volume of sterile DDW shortly before use.

Sterile normal rabbit serum (Lot #53732) was obtained from MBA and stored at -20 C until shortly before use. The normal rabbit serum (NRS)

used throughout this study had been obtained by bleeding (via cardiac puncture) a single group of inbred New Zealand white rabbits; the serum was extracted from the clotted blood by conventional procedures. No additives or preservatives were present in the serum.

10. Salt Solutions

A number of salt solutions were used in this study in an attempt to define the response of CTFV to a variety of organic and inorganic ions and buffers. All salt solutions were prepared as molar concentrations in DDW and sterilized by autoclaving at 10 lb/in^2 for 10 min. Reagent grade chemicals were used exclusively; these were obtained from either Mallinckrodt Chemical Works, St. Louis, Mo., or J. T. Baker Chemical Co., Philadelphia, Pa.

C. Cell Culture Techniques

Several established cell lines were examined in an attempt to determine the most sensitive and suitable host cell for viral assays. Standard cell culture techniques were used for the serial propagation of stock cultures. KB (human carcinoma of nasopharynx), L-929 (malignant mouse fibroblast), Detroit-6 (human sternal marrow), WI-26 (human embryonic lung) and FL (human amnion) were all obtained from MBA. The lines were routinely maintained as fixed cultures on the glass surface of either one-liter Roux flasks or 28-oz prescription bottles. The medium used for routine propagation of stock cell cultures (EBME Ca10) contained EBSS, ten parts of BME (V/V) and one part of sterile calf serum. Incubation of these stock cultures was at 37.5 C in a water-

saturated atmosphere of 95% air and 5% CO_2. Stock cells were maintained in continuous culture for periods up to 90 days; at the end of that time they were discarded, and the line was re-started from frozen reserve cell aliquots (see Appendix for stock cell reserve methods).

Monolayer cultures maintained in the above fashion typically contained 3-8 X 10^7 total cells per flask or bottle. The bottles were ordinarily inoculated with 10^5 - 10^6 cells (hemocytometer total count) dispersed in 50-70 ml of EBME CalO. Fresh medium was added at 72 hr intervals. Under these conditions monolayer confluency was attained in 3-7 days, the time being entirely dependent upon the cell type. The faster growing cells (FL, L-929) easily formed a monolayer in three days when seeded as indicated whereas KB, Detroit-6 and WI-26 cells required 4-7 days to form a monolayer. When the monolayer was complete the cells were removed from the glass surface by a standard trypsinization technique (86). The monolayers were first washed three times with 20 ml of prewarmed (37 C) Saline A. After the final wash the Saline A was removed and replaced with 5 ml of trypsin solution (0.02% in Saline A, also prewarmed to 37 C). The bottle was then returned to the CO_2-air incubator; when cell detachment was complete the cells were gently triturated with a 5-ml pipette, and an equal volume of growth medium was added to inhibit further trypsin activity. A dilution of the cell suspension in growth medium was then prepared and the concentration of cells estimated by direct enumeration in a conventional hemocytometer. The desired split ratio was then calculated, and the diluted cell suspension was dispensed as desired.

D. Tube Dilution Titration of CTFV

A standard tube dilution method (83,86,87,91) for assaying CTFV was used throughout this study. A suspension containing 1-2 X 10^5 cells per ml in EBME CalO was prepared from stock cultures, and 1-ml aliquots were dispensed in Wallis-Melnick (Demuth Glassware, Parkersburg, West Virginia) disposable constricted culture tubes. The inoculated tubes were placed horizontally in suitable racks and incubated overnight in the air-CO_2 mixture. Cell attachment to the glass surface was found to be essentially complete following 12-16 hr of incubation (see Plates 1, 2). Serial ten-fold dilutions of CTFV were prepared in a medium composed of 20 parts of LaH and 1 part of sterile calf serum (LaH Ca5). The medium was removed from the tube cultures, and 1-ml aliquots of the virus/medium mixture were added to each tube. Generally four tube cultures were inoculated with each virus dilution to be tested. Control cultures received 1 ml of the same medium but no virus. All tubes were then tightly stoppered and incubated at 35 C. After 48 hr of incubation the medium was replaced with fresh LaH Ca5 containing twice the usual amount of sodium bicarbonate (1 ml of 7.5% $NaHCO_3$ per 100 ml of medium). The cultures were examined for virus-associated cytopathology 3 and 5 days following virus inoculation. Virus was assumed to be present in the inoculum when, after 5 days, greater than 50% of the cells displayed typical arbovirus cytopathology (see Plates 3,4). Infectivity titers were calculated as $CCID_{50}$/ml using the Reed and Muench method of interpolation (98). A standard method of error estimation (99) was used to evaluate experimental data.

E. Thermal Inactivation Studies

The effect of elevated temperature on CTFV in the presence and absence of NRS and several ionic diluents was examined. In these studies stock CTFV was diluted 1:10 in the diluent under study; aliquots (0.5-1.0 ml) of the virus/diluent mixture were dispensed in conventional serology (hemagglutination) tubes and immersed in a waterbath pre-set at the desired temperature. At given intervals the tubes were withdrawn and immediately immersed either in an icebath or in 95% ethanol at -70 C. The virus/diluent mixture was either immediately assayed for infectivity or stored at -70 C until ready for use.

F. Sterility Testing

Stock cell cultures and virus stocks were routinely cultured for the presence of adventitious bacterial and mycoplasmal contaminants; such sterility testing was performed whenever deemed necessary, usually at approximately 30-day intervals. Media used for sterility testing included sodium thioglycollate, heart infusion broth, PPLO broth, extract agar and BYE agar semi-solid media. Cultures or virus stocks deemed contaminated were discarded.

RESULTS

The Pizzi method of error estimation (99) was used throughout to evaluate the data reported in this study. Under the conditions employed (tenfold virus dilution interval, 4-6 samples per dilution), the calculated standard error is equal to or less than ± 0.27 log units (±47%). A degree of error of this magnitude is entirely compatible with the precision limits of a 50% endpoint determination assay method, wherein the minimum inherent technical error is considered as ± 50%. Obviously, error of tolerable proportions is assumed only under certain circumstances; i.e., 1) only high-titered virus stocks (10^6 or more $CCID_{50}$/ml) are employed, and 2) infectivity changes, rather than absolute titers, are sought.

Cell Sensitivity Studies

Five stable, commercially available cell culture lines were examined for sensitivity to CTFV, strain GS-20 (hereafter referred to simply as CTFV). The five lines tested were L-929, FL, WI-26, KB and Detroit-6 (Det-6). Cell line derivation, culture and assay techniques are discussed in the Materials and Methods section of this study. In these experiments, monolayer cultures were inoculated with tenfold serial dilutions of a single aliquot of stock CTFV (a 1% w/v infected mouse brain homogenate in NRS). A repeat confirmatory experiment was performed, using a second aliquot of the same stock virus preparation. The "apparent" titer of the virus suspension, an index of cell type susceptibility or sensitivity, for each line is given in Table 1 below.

The results given are averages of these two determinations.

Table 1

Cell Line Sensitivity to CTFV

Cell Line	Apparent $CCID_{50}/ml$*
L-929	$10^{9.59}$
FL	$10^{9.67}$
WI-26	$10^{8.20}$
KB	$10^{7.42}$
Det-6	$10^{6.92}$

*For ease of reading and interpretation, whole exponential functions will be used wherever possible to express infectivity titers.

L-929 and FL cells were found to be essentially equal in sensitivity to the virus. The L-929 cell, by reason of its faster growth rate, greater general hardiness and ease of culture, was chosen as host cell for all subsequent determinations.

Effect of Serum Inactivation on the Infectivity of CTFV

In order to optimize the cell culture assay technique for CTFV, the effect of calf serum inactivation (treatment at 56 C for 30 min) on cell culture infectivity of CTFV was determined. Maintenance medium (LaH Ca5) was prepared, incorporating either inactivated or untreated sterile calf serum. The approximate titers of CTFV aliquots diluted in LaH Ca5, prepared in these two ways, were compared. Virus infectivity was determined in the usual manner. The results indicated that there

was no essential difference in infectivity titers ($10^{9.50}$, $10^{9.59}$ $CCID_{50}$/ml) yielded by viral aliquots assayed by these two procedures. Untreated serum was therefore incorporated in all maintenance media subsequently prepared.

Determination of the Cytotoxicity of Normal Mouse Brain Preparations

Since the virus preparations used throughout this study were of mouse brain origin, the overt cytotoxicity of such mouse brain preparations was tested. A normal (uninfected) 1% suckling mouse brain homogenate in NRS was prepared by the same methods used for stock virus passage. Tenfold dilutions of this control homogenate were made in maintenance medium and applied to cell monolayers in the same manner as used for all other experiments. No evident cytotoxic effect was noted in cell cultures at any of the dilutions tested (10^{-3} through 10^{-7}). In order to control the effect of mouse brain in heat inactivation experiments, aliquots of the normal mouse brain preparation were heated at 50 C for 30, 60 and 90 min. Again, 10^{-3} through 10^{-7} dilutions in maintenance medium were applied to monolayer cell cultures; in no case was there any indication of any toxic effect.

Thermal Decay of CTFV at 37 C

As an initial test of virus stability, a single ampoule of stock CTFV was thawed and diluted tenfold in NRS. Eleven aliquots were prepared from this virus suspension and heated for various periods at 37 C (constant-temperature waterbath). Following heating, each aliquot was immediately quick-frozen at -70 C and stored at that temperature until

assayed for infectivity. All aliquots were assayed in a single experiment. The resulting titers and an indication of "per cent infectivity retained" following heating are given in Table 2.

Table 2

Thermal Decay of CTFV at 37 C

Aliquot	Length of Treatment at 37 C	Resulting Titer ($CCID_{50}$/ml)	% Infectivity Retained
A	None	$10^{9.00}$	100
B	4 hrs	$10^{8.57}$	47
C	8 hrs	$10^{9.00}$	100
D	12 hrs	$10^{8.50}$	32
E	16 hrs	$10^{8.50}$	32
F	20 hrs	$10^{8.33}$	21
G	24 hrs	$10^{8.33}$	21
H	28 hrs	$10^{8.33}$	21
I	32 hrs	$10^{8.00}$	10
J	36 hrs	$10^{7.67}$	5
K	92 hrs	$10^{5.50}$	0.03

Even the briefest consideration of the above data indicates the relative imprecision of the tube dilution assay method, at least under the above conditions. Aliquots A and C, D and E, F, G and H, are indistinguishable by this technique. In order to obtain a more significant thermal inactivation rate (stepwise decrease in titer within a practicable period of time), studies were performed in which viral samples were treated at 50 C.

Thermal Decay of CTFV at 50 C

The M57E7M11 CTFV stock lot was examined for thermal response at 50 C (constant-temperature waterbath) in a series of replicate experiments. The results are summarized in Table 3 below and indicated graphically in Figure 1. The data are expressed as calculated averages of four independent determinations, each internally controlled (control titer adjusted to 100%).

Table 3

Thermal Decay of CTFV (M57E7M11 Pool) at 50 C

Time of Heating (Min. at 50 C)	% Infectivity Retained
0	100
5	51.0
10	12.5
15	3.2
20	1.2
25	0.30
30	0.85
35	0.46
40	0.28
45	0.66
50	0.22
55	0.21
60	0.15

In Figure 1 the data have been interpreted as constituting a two-component semi-logarithmic straight line function. The method of Least Squares (100) was used to graphically locate the function. Only by assuming a two-component decay plot can a suitable y-intercept (100% infectivity, 0 time of heating) be obtained. If, for example, the data are presumed to fit a single component semi-logarithmic plot, the y-intercept resulting from Least Squares interpretation is approximately 10%. The fact that the intercept is slightly greater than 100% may indicate that there is a short "lag" in the inactivation process during which the temperature of the virus mixture is increasing from 2-4 C to 50 C.

Certain kinetic information can be calculated from the data in Figure 1. The specific reaction rate (k) of each component can be obtained by inserting suitable values in the kinetic equation for a reaction of the first order:

$$k = \frac{2.303}{t} \log \frac{C_0}{C} \qquad \text{(Eq. 4)}$$

where t is the time of reaction in seconds, C_0 is any initial relative concentration or titer and C is the relative concentration or titer after time t has elapsed. In the case of the first (fast) component in Figure 1:

t = 900 seconds (5 minutes to 20 minutes)

C_0 = 38%

C = 1.2%

Therefore Equation 4 becomes:

$$k = \frac{2.303}{900} \log \frac{38}{1.2}$$

$$\text{or:} \quad k = 0.00256 \times 1.51$$

$$\text{thus:} \quad k = .004 \text{ sec}^{-1}$$

which indicates that during the first twenty minutes of heating (excluding the 0-5 minutes "lag" phase), the virus preparation is undergoing heat inactivation at the rate of 0.4% per second.

The calculated half-life of the virus suspension is obtained by inserting the derived velocity constant k in the following equation:

$$t_{\frac{1}{2}} = \frac{.693}{k} \qquad \text{(Eq. 5)}$$

$$\text{in this case:} \quad t_{\frac{1}{2}} = \frac{.693}{.004 \text{ sec}^{-1}}$$

$$\text{thus:} \quad t_{\frac{1}{2}} = 173 \text{ seconds or 2 minutes, 53 seconds}$$

This value is readily verified by examination of the function illustrated in Figure 1.

The same sort of calculations can be applied to the second (slower) component:

$$t = 1200 \text{ sec (reaction from 25-45 min)}$$

$$C_0 = 0.56\%$$

$$C = 0.30\%$$

$$\text{therefore:} \quad k = \frac{2.303}{1200} \log \frac{.56}{.30}$$

$$\text{so: } k = 0.00192 \times .272$$

$$\text{thus: } k = .0005 \text{ sec}^{-1}$$

which indicates that during the second phase of inactivation the virus suspension is undergoing inactivation at the rate of only 0.05% per second. Employing Equation 5:

$$t_{\frac{1}{2}} = \frac{.693}{.0005 \text{ sec}^{-1}}$$

$$t_{\frac{1}{2}} = 1186 \text{ seconds or } 19 \text{ minutes, } 46 \text{ seconds}$$

The half-life of the virus surviving the first 25 minutes is thus very nearly 20 minutes.

A comparison of the half-lives of the viral populations existing after 5 and 30 minutes of heating indicates the relative stabilities of the two virus "types." Virus surviving the initial rapid decay process is approximately seven times more stable than the original population.

A second CTFV passage pool (M57E7M12) was examined for thermal response at 50 C in the same manner as indicated above. The results, given in Table 4, are calculated averages of four independent experiments in which the control (unheated) virus titer has been adjusted to 100%.

The data are graphically represented in Figure 2. The method of Least Squares (100) was, once again, employed to place the function. Again a two-component function has been indicated. It should be noted that the y-intercept of a single-component plot would be about 88%, an

Table 4

Thermal Decay of CTFV (M57E7M12 Pool) at 50 C

Time of Heating (Min. at 50 C)	% Infectivity Retained
0	100
5	100
10	31.5
15	36.0
20	10.0
25	9.4
30	10.0
35	21.5
40	6.9
45	1.5
50	1.5
55	1.9
60	0.76
65	0.96

approximate error of 12%. Given the relative imprecision of the tube dilution assay method, either a one- or two-component interpretation is obviously possible. The greater stability of the fast component of the M57E7M12 virus pool is indicated by the calculated velocity constants (k) and half-lives of the components:

Table 5

Calculated Decay Kinetics of CTFV, Pool M57E7M12

Component	Velocity Constant (k)	Half-Life
Fast (3-16 min.)	.0018 sec^{-1}	385 seconds
Slow (20-60 min.)	.0011 sec^{-1}	630 seconds

Effect of Several Cations Upon CTFV

Experiments were performed in which a wide variety of cations were tested for their effect on CTFV (Lot M57E7M12). One molar solutions of the sulfate or chloride salts were prepared and used as diluents for the virus. The virus/salt suspensions were heated at 50 C for 30 minutes, quick-frozen at -70 C, and later assayed for infectivity. The final salt concentrations in the heated preparations were 0.9 M. At the time of virus addition, considerable precipitate was noted in most cases. The precipitates were resuspended by agitation at intervals during the heating and immediately before assay so that relatively even distribution was assured. Virus/salt suspensions were sufficiently diluted (ten-thousandfold) in maintenance medium (LaH Ca5) before assay to preclude any salt effect upon the tissue culture assay system. No attempt was made to resolve inactivation greater than 99.9% (0.1% infectivity retained), as this constitutes the practical limits of the assay system. A control titration of unheated virus (in NRS) served as a baseline (100% infectivity) for the calculated decay data given in Table 5.

The results indicate rather simply that large amounts of most cations cause the rapid inactivation of CTFV. The exception is Na^+

and perhaps K^+). Salts of these cations were not as effective as serum in stabilizing the virus, but were notably better than distilled water as a diluent.

Effect of Sodium and Potassium Citrate and Sodium Phosphate on CTFV

Two salts commonly used in arbovirus purification methods were tested for their effect on CTFV. The sodium and potassium salts of citric acid and the disodium salt of phosphoric acid were tested both at neutral pH and at alkaline pH (commonly employed for arbovirus work). The use of the sodium and potassium cations was clearly indicated by the data of Table 6. The stock virus was diluted 1:10 in the respective salt and the virus/salt suspension split into two aliquots. One aliquot was maintained at 0 C and the other was heated for 30 min at 50 C. The results, expressed as per cent infectivity retained after heating, are illustrated in Table 7. Data for decay of CTFV in the presence of normal rabbit serum (NRS) and DDW are included as a ready reference. The final concentration of the salt in all cases is 0.9 M. Salt/virus suspensions were diluted ten-thousandfold in LaH Ca5 before assay to preclude any specific salt effect on the cell culture assay system.

The data in Table 7 indicate that stock CTFV (1% infected mouse brain homogenate in NRS) diluted tenfold in 1.0 M sodium or potassium citrate is significantly more stable at 50 C than virus in NRS alone. The effect appears to be relatively independent of pH, since good stabilization is obtained at both neutral and alkaline pH. Disodium

phosphate, on the other hand, functioned poorly as a stabilizing diluent.

Table 6

Effect of Salts as Diluents for CTFV

Diluent	% Infectivity Retained*
NRS	10.0
DDW**	< 0.1
$AlCl_3$	< 0.1
$BaCl_2$	< 0.1
$CaCl_2$	< 0.1
$CoCl_2$	< 0.1
$CuCl_2$	< 0.1
$FeCl_2$	< 0.1
KCl	1.5
$MgSO_4$	< 0.1
$MgCl_2$	< 0.1
$MnCl_2$	< 0.1
Na_2SO_4	5.0
NaCl	0.4
$NiCl_2$	< 0.1
$ZnCl_2$	< 0.1

*Following 30 min at 50 C; difference from unheated CTFV titer.

**Double distilled and deionized water.

Table 7

Effect of Citrate and Phosphate Buffers on Infectivity of CTFV

Diluent	pH	% Infectivity Retained*
NRS	7.2	10.0
DDW	6.6	< 0.1
1.0 M Na_2HPO_4	8.9	0.5
Na_2HPO_4/NaH_2PO_4, 1.0 M	7.0	1.0
1.0 M $Na_3C_6H_5O_7$	8.5	18.6
$Na_3C_6H_5O_7/H_3C_6H_5O_7$, 1.0 M	7.0	14.8
1.0 M $K_3C_6H_5O_7$	8.2	17.8
$K_3C_6H_5O_7/H_3C_6H_5O_7$, 1.0 M	7.0	14.8

*Per cent of original (unheated) infectivity remaining after heating at 50 C for 30 min.

Effect of pH on Thermal Stability of CTFV

In order to insure that the observed stabilization of CTFV in the presence of sodium and potassium citrate is not merely an artifact of pH, thermal decay experiments were carried out on virus in the presence of borate-saline buffer (0.05 M borate, 0.12 M NaCl), pH 9.0, and Tris-HCl buffer (trihydroxymethyl aminomethane, 1.0 M) at pH 8.6. The results are given in Table 8; also tabulated is the response of the virus in the presence of several other diluents encompassing a wide pH range. Results are expressed as "per cent infectivity retained"; the infectivity of control preparations (titer of unheated virus preparations

containing respective diluent) was assigned a value of 100% in each case.

Table 8

Effect of pH on Thermal Stability of CTFV

Diluent	pH	% Infectivity Retained*
1.0 M NaCl	5.4	0.4
1.0 M KCl	5.6	1.5
Na_2HPO_4/NaH_2PO_4, 1.0 M	7.0	1.0
$K_3C_6H_5O_7/H_3C_6H_5O_7$, 1.0 M	7.0	14.8
$Na_3C_6H_5O_7/H_3C_6H_5O_7$, 1.0 M	7.0	14.8
NRS	7.2	10.0
1.0 M $K_3C_6H_5O_7$	8.2	17.8
1.0 M $Na_3C_6H_5O_7$	8.5	18.6
1.0 M Tris-HCl	8.6	< 0.1
1.0 M Na_2HPO_4	8.9	0.5
Borate-Saline:		
0.05 M Borate 0.12 M NaCl	9.0	< 0.1

*Per cent of original (unheated) infectivity remaining after heating at 50 C for 30 minutes.

Although maximum viral stability is evident in the pH 7-8.5 range, the response of the agent to Tris-HCl, phosphate (either at neutral or alkaline pH) and borate-saline buffers indicates that the observed stability in the presence of citrate is not simply a pH artifact.

The Effect of Potassium Citrate Concentration on CTFV Stability at 50 C

The effect of varying concentrations of potassium citrate (pH 8.2) was investigated. Salt solutions were prepared at 2.0, 1.0, 0.1, 0.01 and 0.001 M concentrations. An aliquot of stock CTFV was thawed and diluted (1:10) in the various preparations. For each salt concentration, one heated (30 min, 50 C) and one control (unheated) suspension were assayed. Preparations were diluted ten-thousandfold prior to assay, thus precluding any effect of the salt on the cell culture assay system. The results are given in Table 9.

Table 9

Effect of Potassium Citrate Concentration on CTFV at 50 C

Concentration of $K_3C_6H_5O_7$	% Infectivity Retained*
0.001 M	< 0.1
0.01 M	< 0.1
0.1 M	12.0
1.0 M	21.4
2.0 M	67.8

*Per cent of original (unheated) infectivity remaining after heating at 50 C for 30 min.

In the presence of 2.0 M potassium citrate, the virus of Colorado tick fever, GS-20 strain, is extremely stable, losing only some 32% infectivity under the conditions employed.

Comparison of Potassium Citrate, NRS and DDW as Diluents for Freeze-Thaw Manipulation of CTFV

One molar potassium citrate (pH 8.2), normal rabbit serum (NRS) and distilled water (DDW) were compared as diluents for CTFV subjected to repeated freezing and thawing. An aliquot of stock virus was thawed (this thawing was not counted in the determination) and diluted 1:10 in each of the diluents. A portion of each preparation was set aside (0 C) for immediate assay; the remaining virus suspensions were alternately quick-frozen in ethanol at -70 C and thawed in a 37 C waterbath five times. Following the fifth cycle, all preparations were assayed for infectivity. The results are given in Table 10.

Table 10

Freeze-Thaw of CTFV Diluted in Potassium Citrate, NRS, and DDW

Diluent	Freeze-Thaw Cycles	Titer*	% Infectivity Retained
DDW	- - -	$10^{9.00}$	
DDW	5	$<10^{6.00}$	< 0.1
NRS	- - -	$10^{10.00}$	
NRS	5	$10^{10.00}$	100
1.0 M $K_3C_6H_5O_7$	- - -	$10^{10.33}$	
1.0 M $K_3C_6H_5O_7$	5	$10^{9.67}$	21.8

*As $CCID_{50}$/ml

It is evident that NRS functioned as the most efficient protective diluent tested for freeze-thaw manipulations. One molar potassium

citrate offers a significant protective effect, while distilled water allows the loss of greater than 99.9% of the virus infectivity under the conditions described.

Electron Microscopy of CTFV Preparations

Two CTFV suspensions, each containing approximately 10^6 $CCID_{50}$/ml, were prepared by dilution of stock virus (M57E7M12 pool) one-hundredfold either in NRS or 1.0 M potassium citrate. Control solutions, containing diluent only, but no virus, were also prepared. These virus suspensions and control solutions were given to Irving DeVoe, who performed the following study. Droplets of each suspension were adsorbed onto Parlodion-coated grids (Mallinckrodt Chemicals, St. Louis, Mo.) and evaporated to dryness at room temperature. The grids were shadow-cast at an angle of 17 deg from the horizontal with platinum in vacuo and visualized in an RCA EMU-3F electron microscope at an instrumental magnification of 50,000 X. "Virus-like particles" (50-100 mμ in diameter) were observed in the virus/citrate preparations (see Plates 5-7). No such particles were seen in the virus/NRS suspensions, which appeared to contain considerably more debris, perhaps masking the particles. No similar particles were evident in control preparations.

Citrate as a Primary Infected-Tissue Diluent

In the experiments thus far detailed, 1 M potassium citrate as a diluent was found to function approximately twice as effectively as NRS for protection of CTFV preparations (1% infected mouse brain homogenate in NRS) heated at 50 C. Even in the experiments incorporating citrate

as diluent a small amount, i.e., 10%(v/v), of NRS was always present, since serum was employed as the primary infected tissue diluent.

Since one of the primary aims of this study was to replace NRS as a viral diluent with some low molecular weight material, the next logical step was to incorporate potassium citrate as the primary tissue diluent (effectively by-passing the use of NRS at all). Accordingly, the following series of experiments were initiated.

Suckling mice were inoculated in the usual manner (see Materials and Methods) with an aliquot of the M57E7M12 CTFV pool. As the mice demonstrated encephalitic involvement, they were killed and stored as usual, at -70 C. The infected animals were then divided randomly into two groups. From the first group a 1% brain homogenate in NRS was prepared as usual. From the second group a 1% homogenate was prepared using 1.0 M potassium citrate as system diluent.

The 1% virus suspension in NRS was designated the "A" preparation; the homogenate prepared using citrate alone was designated as the "B" virus lot. A series of experiments were then initiated, comparing the "A" and "B" virus source material.

Comparative Stabilities of "A" and "B" Virus Preparations at Various Temperatures

Paired aliquots of "A" and "B" virus preparations were subjected to temperature-stability studies at -70, 4, 23, 37 and 50 C. At the indicated intervals, virus suspensions were assayed for infectivity (or quick-frozen at -70 C for later assay) in the usual manner, pre-diluting

all suspensions in LaH Ca5 sufficiently to preclude a cellular toxic effect. The results of these comparative experiments are detailed in Table 11. The indicated "per cent infectivity retained" estimates are averages of replicate and independent determinations.

It is apparent from the data in Table 11 that the use of potassium citrate alone as a virus diluent yields a preparation significantly more labile at 50 C than a corresponding virus suspension diluted only in NRS.

Reversibility of the NRS and Citrate Effects

Studies were performed to determine whether the heat lability displayed by the "B" virus preparation could be readily reversed by the simple addition of NRS to "B" material. An aliquot of "A" virus (1% infected mouse brain homogenate in NRS) was diluted tenfold in 1.0 M potassium citrate. To 0.5 ml of "B" virus (1% infected mouse brain homogenate in molar potassium citrate) were added 4.0 ml of 1.0 M potassium citrate and 0.5 ml NRS. The compositions of the two preparations described were therefore made as nearly identical as possible. Both were then heated at 50 C for varying times; infectivity determinations were performed as usual. If the adverse effects of citrate in the "B" preparation were, in fact, readily reversible, then the decay kinetics of the two suspensions just described should be very nearly equal. The results of this experiment are given in Table 12.

The adverse changes evoked in CTFV upon dilution of infected mouse brain material in citrate are evidently irreversible, or at least not reversible by simple dilution into a mixture containing NRS. In a similar experiment NRS was added to a "B" virus suspension after 10 min

Table 11

Comparison of Stability of "A" and "B" Virus Preparations at Various Temperatures

Treatment Temperature	Length of Treatment	% Infectivity Retained "A"*	% Infectivity Retained "B"**
-70 C	- -	100	100
	60 days	100	68
	120 days	100	52
	180 days	100	32
4 C	- -	100	100
	24 hr	n.t.***	27
	36 hr	68	n.t.
	72 hr	46	15
	164 hr	46	10
	216 hr	33	10
23 C	- -	100	100
	4 hr	68	34
	16 hr	68	26
	36 hr	68	n.t.
	48 hr	n.t.	0.3
	72 hr	32	<0.1
	140 hr	3.3	<0.1
37 C	- -	100	100
	1 hr	48	5.0
	3 hr	n.t.	4.6
	4 hr	15	n.t.
	16 hr	12	<0.1
	46 hr	2.3	<0.1
50 C	- -	100	100
	10 min	13	10
	13 min	n.t.	0.7
	20 min	0.9	<0.1
	40 min	0.5	<0.1

* "A" - 1% (w/v) mouse brain homogenate in NRS
** "B" - 1% (w/v) mouse brain homogenate in 1.0 M potassium citrate
*** n.t.- not tested

Table 12

Effect of Serum Addition on "B" Virus

Preparation	Time of Heating	% Infectivity Retained*
	- -	100
0.5 ml "A" + 4.5 ml	10 min	46
1.0 M $K_3C_6H_5O_7$	20 min	66
	40 min	22
	- -	100
0.5 ml "B" + 0.5 ml	10 min	2.1
NRS + 0.5 ml	20 min	0.46
$K_3C_6H_5O_7$	40 min	0.31

*Per cent of original (unheated) infectivity remaining after heating for the indicated period of time.

of heating at 50 C. The addition of serum had no observable effect on the rate of thermal decay; i.e., the extent of inactivation was approximately the same whether NRS or more citrate (to control dilution effect) was added.

Effect of Serum Fractions on Thermal Stability of CTFV

It appeared possible that a serum fraction might evoke a significant stabilizing influence on the virus. Whole NRS (20-ml volume) was therefore fractionated by conventional ammonium sulfate precipitation. The fractions were subjected to dialysis vs 0.85% NaCl, and the volume of

each was adjusted to 20 ml. The following serum fractions were tested for their effect on CTFV heated at 50 C. The usual assay method was followed.

Table 13

Serum Fractions Prepared for Test of Effect on CTFV at 50 C

Fraction	Nature of Fraction
A	Whole NRS
B	0-80% ammonium sulfate fraction
C	Supernatant remaining after removal of 0-80 fraction*
D	Whole NRS dialysate (small molecules escaping dialysis tubing during dialysis of NRS)*
E	0-33% fraction
F	33-40% fraction
G	40-50% fraction
H	50-60% fraction
I	60-80% fraction

*Volume reduced to 20 ml by pervaporation at 4 C.

Only three of the preparations had any significant stabilizing effect on the virus. Whole serum (A) gave the expected result. The 0-80% fraction (B), containing most of the protein found in whole serum, also has a significant stabilizing effect. Fraction F, the 33-40% fraction, also appeared to stabilize the virus to an extent nearly equal that of Fraction B. This was of particular interest since a biuret

Table 14

Effect of Serum Fractions on Stability of CTFV at 50 C

Fraction	Time of Heating (50 C)	% Infectivity Retained
A	- -	100
	15 min	55
	30 min	7.6
B	- -	100
	15 min	10
	30 min	1.0
C	- -	100
	15 min	0.2
	30 min	<0.1
D	- -	100
	15 min	<0.1
	30 min	<0.1
E	- -	100
	15 min	1.0
	30 min	<0.1
F	- -	100
	15 min	6.9
	30 min	0.5
G	- -	100
	15 min	0.8
	30 min	<0.1
H	- -	100
	15 min	0.2
	30 min	<0.1
I	- -	100
	15 min	0.4
	30 min	<0.1

protein determinations disclosed that Fraction F contained only about 1% (1.2 mgm protein/ml) of the protein found in whole NRS (124 mgm/ml). Fraction F was therefore examined again, this time in comparison with whole NRS and NRS diluted one-hundredfold to approximately the same protein concentration (1.2 mgm/ml). The results of this experiment are given in Table 15.

Table 15

Comparison of NRS, Diluted NRS and Fraction F on the Thermal Stability of CTFV

Diluent	Protein Concentration	% Infectivity Retained
NRS	120-125 mgm/ml	28
1:100 NRS in .85% NaCl	1.2 mgm/ml	3.2
Fraction F (33-40% ammonium sulfate fraction)	1.2 mgm/ml	6.8

*After 15 min at 50 C; per cent of unheated original (control) infectivity.

The above data indicate that CTFV may be stabilized principally by a non-specific protein effect, rather than by any particular specific fraction or fraction of normal rabbit serum or its components.

The Effect of EDTA, Glycerol, Sucrose, and Glutamine on the Thermal Stability of CTFV

While this investigation was in progress there appeared in the literature (88) a report that glycerol, 20 mM L-glutamine and several

animal sera stabilized the Florio strain of CTFV to freeze-thaw manipulation. It was decided to test such preparations for their effect on CTFV at 50 C. The following solutions were therefore prepared:

1.0% Ethylenediaminetetracetic acid (EDTA)

0.1% EDTA

0.01% EDTA

50% glycerol in phosphate-buffered saline, pH 7.0

1.0 M sucrose

20 mM L-glutamine

Each of the above was tested as a diluent for CTFV heated at 50 C. A portion of the M57E7M12 stock pool was thawed, and aliquots were diluted tenfold in each diluent. The resulting virus suspension was immediately assayed for infectivity, and an aliquot was heated at 50 C for 30 min prior to assay. Virus titration was performed as usual. In all cases incorporating any of the above solutions as diluents, more than 99.9% of the control (unheated) infectivity was lost during the 30-min heating period. Furthermore, in order to determine whether the protective effect demonstrated by citrate in the presence of NRS might result from ion complexing, it was decided to evaluate the stabilizing effect of a known chelating agent, EDTA, on CTFV heat stability.

DISCUSSION

This study appears to be the first such investigation of any arbovirus in which both the kinetics of heat inactivation and viral response to small molecules have been examined simultaneously and in some detail. It is also the first adequate test of the theory of Wallis and Melnick (61) as it applies specifically to the arbovirus group. Wallis and Melnick found that Sindbis virus (a group A arbovirus) was not stabilized at 50 C by dilution in 1 M $MgCl_2$. Their conclusion from this single determination was that the arbovirus group was probably characterized by such a response to divalent cations. The results of this investigation apparently confirm Wallis' and Melnick's concept, in that no cation tested exerted a marked protective effect upon CTFV, strain GS-20. Indeed, all of the salts tested (with the notable exception of sodium and potassium citrate) had precisely the opposite effect, that of significantly enhancing the thermal decay rate. Wallis and Melnick would probably attribute this response to the presence in or on the virus of lipid essential for infectivity and the probable lack of icosahedral (cubic) symmetry.

The role of a citrate salt in the presence of normal rabbit serum in stabilizing CTFV is unclear. It apparently does not function as a simple chelating agent, since EDTA had no similar effect. One cannot rule out the possibility of a combined pH and chelating effect; the low pH of EDTA precludes a direct comparison of the two variables. It is clear, however, that citrate alone offers no significant sparing effect; the citrate stabilizing influence was present only when 10% v/v serum was also present in the virus suspension.

It has been the common practice of investigators engaged in virus kinetic studies, which are essentially examinations of biological events, to extract from their data such calculated physical/chemical values as energy and entropy of activation, and to infer from them the nature of the molecular decay processes (10, 13, 16, 20, 101). Given the imprecision of any present virus assay or quantitating system, the writer questions the significance of such interpretations of kinetic data, particularly when the product of those interpretations is an elaborate theory purporting to clearly explain the very molecular basis of the inactivating event.

However, even with these reservations in mind, it seems reasonable to "put the data to work" in order to compare the results of this study with those similarly reported by others. In the following discussion the data are subjected to kinetic analysis with the aim of tentatively identifying the principle decay event in preparations of CTFV exposed to elevated temperatures. The writer asks that the reader bear in mind, however, the limits of the quantitating system used in this or any other virus study and the biological complexity of the viral agent.

A velocity constant (k) was earlier calculated for the inactivation at 50 C of CTFV, stock pool M57E7M11. This constant (.004 sec^{-1}) will here be referred to as k_2. Another rate constant (k_1) can be similarly calculated for the decay of the same preparation at 37 C. In Figure 3, the data of Table 3 have been transposed to a semi-logarithmic plot and the decay function placed by the method of "Least Squares" (100). The resulting curve yields a satisfactory y-intercept (100%) and may be

assumed to be a representative, if not entirely precise, expression of virus decay at 37 C. The specific reaction rate k_1 can be obtained by inserting suitable values extracted from Figure 3 in the kinetic equation for a reaction of the first order:

$$k_1 = \frac{2.303}{t} \log \frac{C_0}{C} \qquad \text{(Eq. 4)}$$

where t is the time of the reaction in seconds, C_0 is any initial relative concentration or titer, and C is the relative concentration after time t has elapsed. Since the reaction apparently yields a single component throughout, C_0 and C values may be picked at any point on the curve.

t = 43,200 sec (reaction from 0-12 hr)

C_0 = 100% (initial concentration)

C = 4.2% (relative concentration after 12 hr)

thus: $k_1 = \frac{2.303}{43200} \log \frac{100}{4.2}$

or: $k_1 = 5.35 \times 10^{-5} \times \log 23.8$

simplifying: $k_1 = 5.35 \times 10^{-5} \times 1.36$

therefore: $k_1 = 7.3 \times 10^{-5}\ \text{sec}^{-1}$

This simply means that at 37 C the virus suspension is decaying at the rate of approximately .007 per cent per second.

Two velocity constants have now been calculated: k_2(specific reaction rate constant at 50 C) and k_1 (rate constant at 37 C). From these two values and the relationship expressed by Equation 6, the energy of

activation of the decay event can be calculated.

$$\log \frac{k_2}{k_1} = \frac{E}{2.303R} \times \frac{T_2 - T_1}{T_1 T_2} \qquad \text{(Eq. 6)}$$

where k_1 and k_2 are the velocity constants described above for the reaction carried out at the absolute temperatures T_1 and T_2; R is the gas constant (1.987 cal deg^{-1} $mole^{-1}$); and E is the energy of activation of the process, usually expressed in calories or kilocalories per mole of reacting substance. Substituting these values in Equation 6, we obtain:

$$\log \frac{4.0 \times 10^{-3}}{7.3 \times 10^{-5}} = \frac{E}{2.303 \times 1.987} \times \frac{323 - 310}{323 \times 310}$$

simplifying: $$\log 54.8 = \frac{E}{4.58} \times \frac{13}{1 \times 10^5}$$

or: $$1.74 = \frac{13 \times E}{4.58 \times 10^5}$$

solving for E: $$E = \frac{1.74 \times 4.58 \times 10^5}{13}$$

therefore: $$E = 6.12 \times 10^4 \text{ cal/mole or } 61.2 \text{ kcal/mole}$$

This value represents a crude approximation of the amount of energy required to evoke the observed reaction; i.e., loss of infectivity. A more precise determination of essentially the same unit can be obtained by use of the Eyring theory of absolute reaction rates incorporating the following identity:

$$\ln k_x = \ln \frac{k T_x}{h} + \frac{\Delta S}{R} - \frac{\Delta H}{R T_x} \qquad \text{(Eq. 7)}$$

In this identity, ΔH, or the heat of activation, has been substituted for E (energy of activation); the two are for practical purposes identical, although a determination of ΔH takes into consideration heat-induced changes in specific volume. The principle value of the Eyring equation is that it allows the calculation of ΔS, the entropy of activation. A knowledge of the ΔS value is of significance since it enables one to infer the difference in ultimate energy levels between the reactants (infectious virus) and the products of the reaction (inactive virus). Differing chemical classes (proteins, DNA, RNA, etc.) are known to have distinct ΔS values when inactivated or denatured by heat. A knowledge of both ΔH and ΔS allows, in theory at least, the calculation of the precise nature of the inactivating event.

In the Eyring equation (Eq. 7), k_x is the specific reaction rate at any absolute temperature T_x; k is Boltzmann's constant (1.380×10^{-16} ergs deg^{-1}); h is Planck's constant (6.625×10^{-27} erg-sec); R is the gas constant (1.987 cal per deg per mole). Since we have calculated both k_1 and k_2, the equation is most simply solved by inserting the proper values into two identities and solving the simultaneous equations for either ΔS or ΔH.

Eyring Equation for Virus Inactivated at 50 C (Eq. A):

$$k_x = 4 \times 10^{-3} = k_2$$

$$\text{therefore: } \ln (4 \times 10^{-3}) = \ln \frac{1.38 \times 10^{-16} \times 323}{6.625 \times 10^{-27}} + \frac{\Delta S}{1.987} - \frac{\Delta H}{1.987 \times 323}$$

$$\text{simplifying: } -5.53 = \ln (67.3 \times 10^{11}) + \frac{\Delta S}{1.987} - \frac{\Delta H}{641}$$

$$\text{or: } -5.53 = 29.6 + \frac{\Delta S}{1.987} - \frac{\Delta H}{641}$$

$$\text{rearranging: } 1.987\Delta H = 641\Delta S + 44{,}700 \qquad \text{(Eq. A)}$$

Eyring Equation for Virus Inactivated at 37 C (Eq. B)

$$k_x = 7.3 \times 10^{-5} = k_l$$

therefore:

$$\ln (7.3 \times 10^{-5}) = \ln \frac{1.38 \times 10^{-16} \times 310}{6.625 \times 10^{-27}} + \frac{\Delta S}{1.987} - \frac{\Delta H}{1.987 \times 310}$$

solving as above: $1.987\Delta H = 616\Delta S + 48{,}800$ (Eq. B)

If the simultaneous equations A and B are solved for ΔS:

$$1.987\Delta H = 641\Delta S + 44{,}700 \qquad \text{(Eq. A)}$$

$$-\ 1.987\Delta H = 616\Delta S + 48{,}800 \qquad \text{(Eq. B)}$$

$$0 = 26\Delta S - 4100$$

therefore: $\Delta S = 164$ cal per mole

Substituting for ΔS and solving for ΔH:

$$1.987\Delta H = 641 \times 164 + 44{,}700$$

$$\Delta H = 75{,}400 \text{ cal/mole or } 75.4 \text{ kcal/mole}$$

The calculated value of ΔH above is similar to that of E, calculated previously. Both the heat (ΔH) and entropy (ΔS) of activation are

seen to be rather large positive values. The only virus-associated component having such thermal decay kinetic equivalents is protein (16). Ribonucleic acid is usually inactivated in a manner yielding a low positive heat of activation and a negative entropy value (20). Deoxyribonucleic acid, not present in CTFV, may display similarly high ΔH and positive ΔS values.

The evidence would indicate, then, that the virus of Colorado tick fever is inactivated at 50 C as the result of some change in the protein component of the virion. This observation is of particular interest in view of Barnes' findings with Sindbis virus.[1] Inactivation of this virus appeared to be the result of opening of the protein capsid, since heated preparations were significantly more sensitive to the effects of RNase. Similar studies with CTFV might prove to be most interesting.

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[1]Barnes, R. Personal Communication, 1967.

APPENDIX

1. Composition of Earle's Balanced Salt Solution (EBSS), 1X (93)

Component	Grams/Liter
NaCl	6.80
KCl	0.40
$MgSO_4 \cdot 7H_2O$	0.20
$NaH_2PO_4 \cdot H_2O$	0.14
Glucose	1.00
$CaCl_2$	0.20
$NaHCO_3$	2.20
Phenol Red	0.01
Distilled H_2O q.s. ad	1 Liter

2. Composition of Basal Medium Eagle (BME) (94,95)

Component	Milligrams/Liter
L-arginine	21.0
L-cystine	12.0
L-tyrosine	18.0
L-histidine	8.0
L-isoleucine	26.0
L-leucine	26.0
L-lysine	26.0
L-methionine	7.5
L-phenylalanine	16.5
L-threonine	24.0
L-tryptophan	4.0

L-valine	23.5
L-glutamine	292.0
Biotin	1.0
Folic Acid	1.0
Choline Cl	1.0
Nicotinamide	1.0
Ca-D-Pantothenate	1.0
Pyridoxal HCl	1.0
Thiamine HCl	1.0
Riboflavin	0.1
i-inositol	1.8

3. Reserve Stock Cell Freezing, Storage and Reconstitution

a) Freezing Procedure

The cell cultures received from MBA were split and subcultured (see Materials and Methods for procedure) as soon as possible after arrival. When the resulting cultures had reached approximately 90-95% full surface coverage (90-95% monolayer), they were processed as follows. The growth medium, EBME Cal0, was removed from the bottles by aspiration, and fresh pre-warmed (37 C) EBME Cal0 was added (5-10 ml per 28-oz prescription bottle or Roux flask). The cells were scrubbed from the surface with the aid of a sterile rubber policeman and dispersed by gentle pipette trituration. The resulting cell suspensions were pooled and centrifuged at 4 C for 15 min at 500 rpm. The supernatant was decanted from the pelleted cells, and the cells were

resuspended with gentle agitation in fresh EBME CalO containing 10% (v/v) sterile glycerol to approximately one-tenth the original culture volume; i.e., 5-7 ml per bottle or flask harvested. The dispersed cell suspension was dispensed in convenient aliquots (usually 2 ml) into sterile 2- or 5-ml ampoules. The ampoules were flame sealed. Slow freezing was accomplished by 1) placing the sealed ampoules in a conventional refrigerator at 4 C for 2-4 hr, 2) transferring to an upright freezer (-10 C) for 2-4 hr, and finally 3) transferring the ampoules to the Revco chest freezer (-70 C) for storage.

b) Reconstitution Procedure

The frozen cell aliquots were rapidly thawed by immersion in running tap water at 40 C and gentle shaking. The cells were added to an equal volume of pre-warmed (37 C) EBME CalO; the suspension was evenly distributed over the surface of the desired culture vessel and incubated at 37 C in a water-saturated atmosphere of 95% air and 5% CO_2. At approximately 2-hr intervals, the volume of the cell suspension was doubled by addition with gentle mixing of fresh pre-warmed (37 C) EBME CalO, until the desired final volume (50-70 ml) was achieved. The cell cultures were then incubated at 37 C in the air/CO_2 atmosphere as usual.

Figure 1

Thermal inactivation of the M57E7M11 passage pool of CTFV, GS-20 strain, at 50 C.

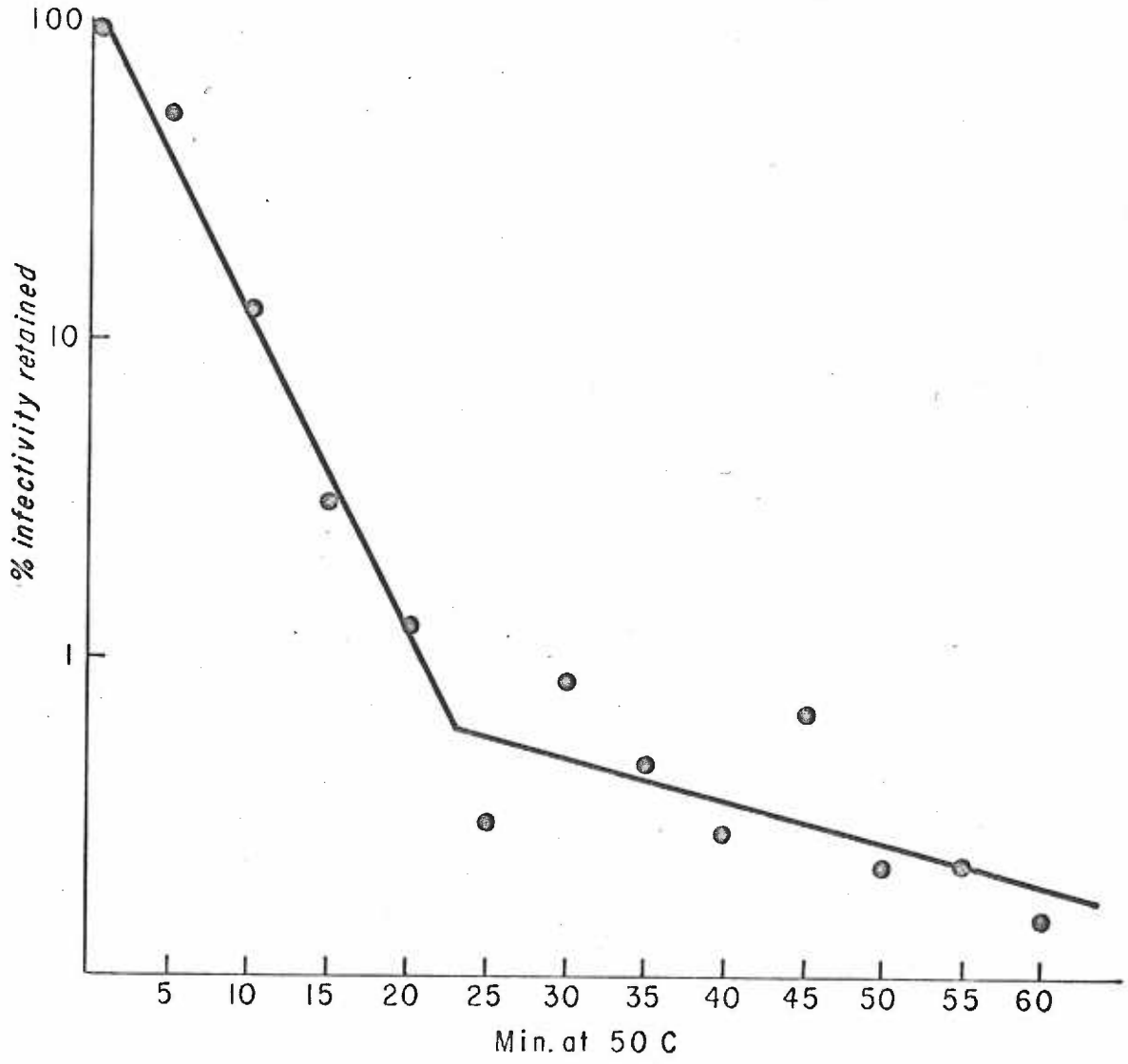

100
10
1
% infectivity retained
5
10
15
20
25
30
35
40
45
50
55
60
Min. at 50 C

Figure 2

Thermal inactivation of the M57E7M12 passage pool of CTFV, GS-20 strain, at 50 C.

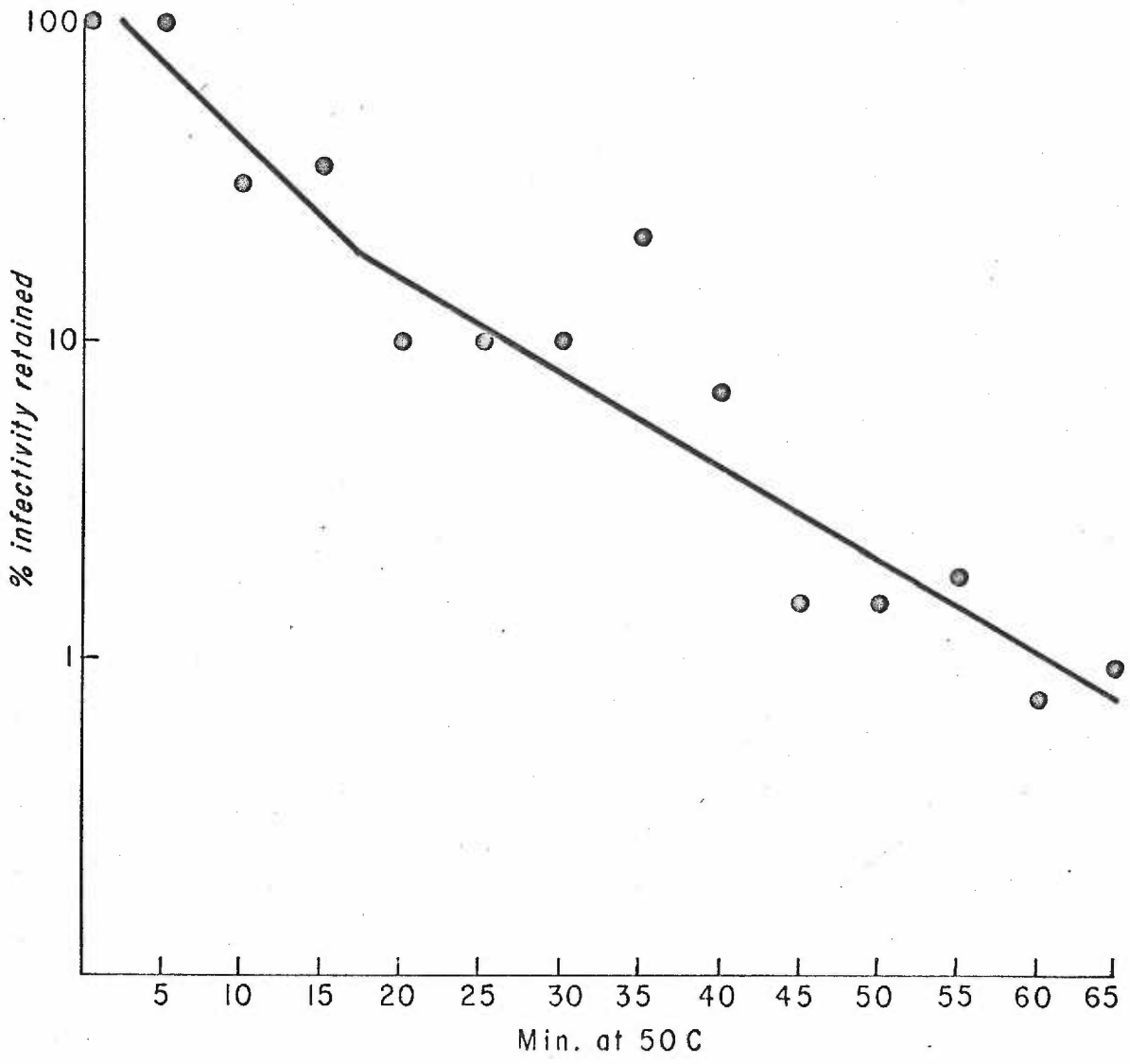

100
10
1
% infectivity retained
5
10
15
20
25
30
35
40
45
50
55
60
65
Min. at 50 C

Figure 3

Thermal inactivation of CTFV at 37 C.

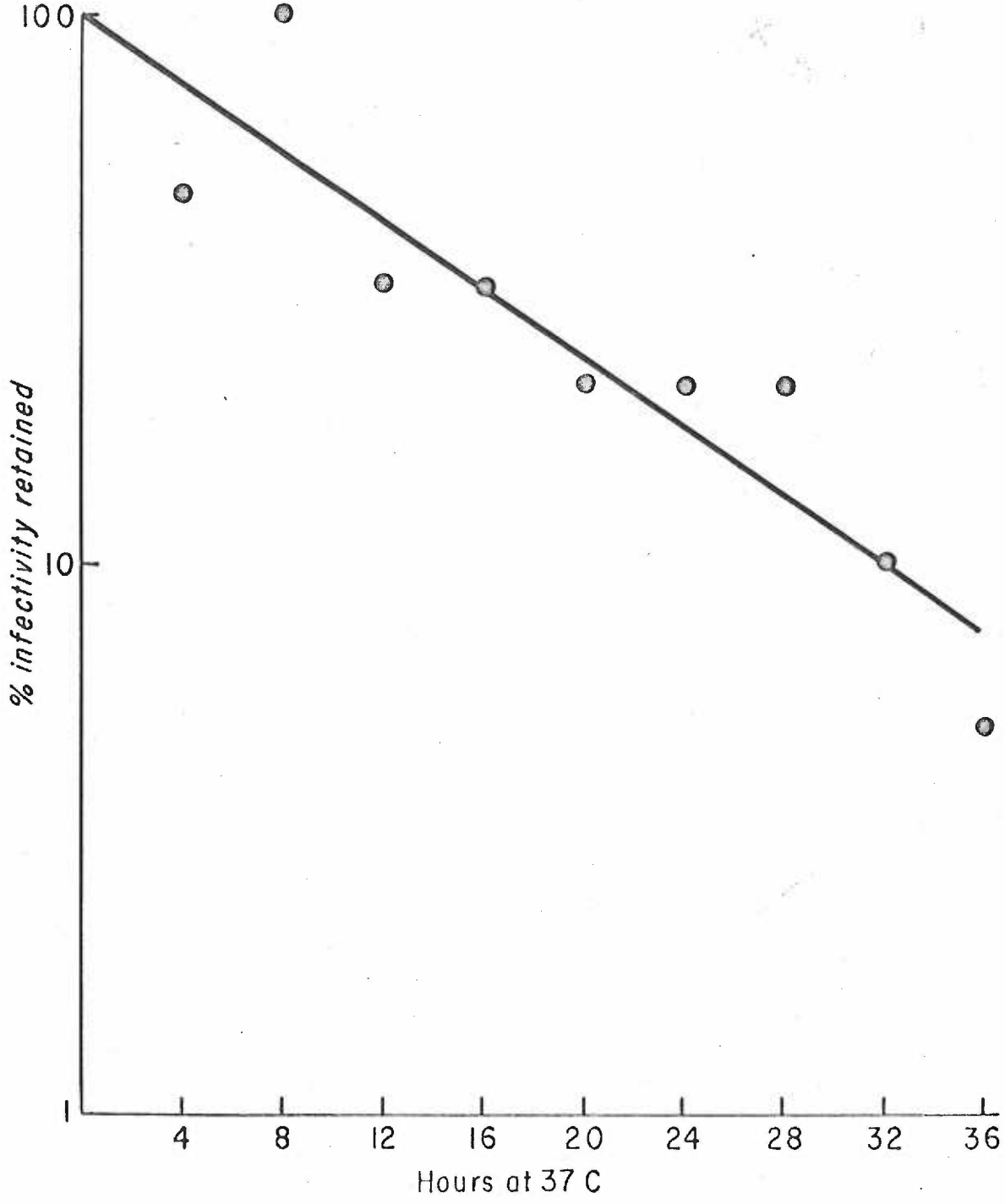

100
10
1
% infectivity retained
4
8
12
16
20
24
28
32
36
Hours at 37 C

Plate 1

L-929 cell density at time of virus inoculation; attached cell appearance 24 hr after seeding Wallis-Melnick culture tube with 2×10^5 cells. (950X)

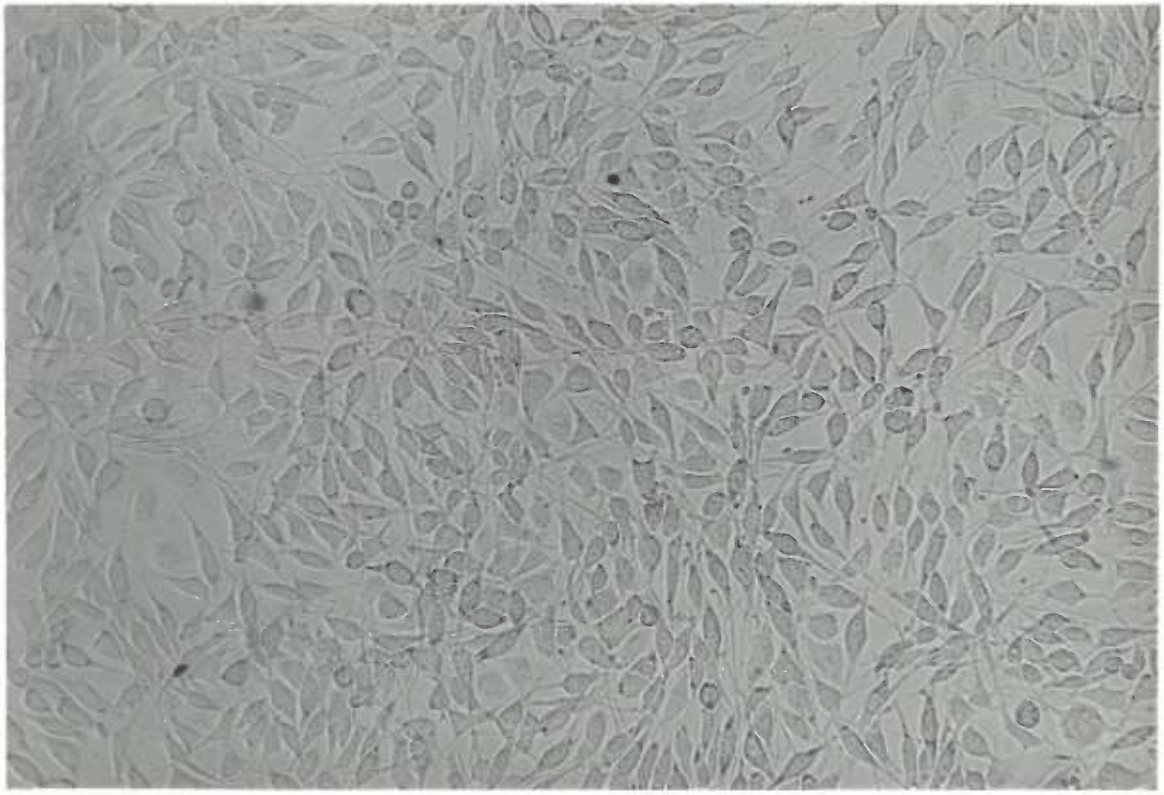


Plate 2

Normal (uninfected) L-929 cell monolayer 72 hr after seeding Wallis-Melnick culture tube with 2 X 10^5 cells. (950X)

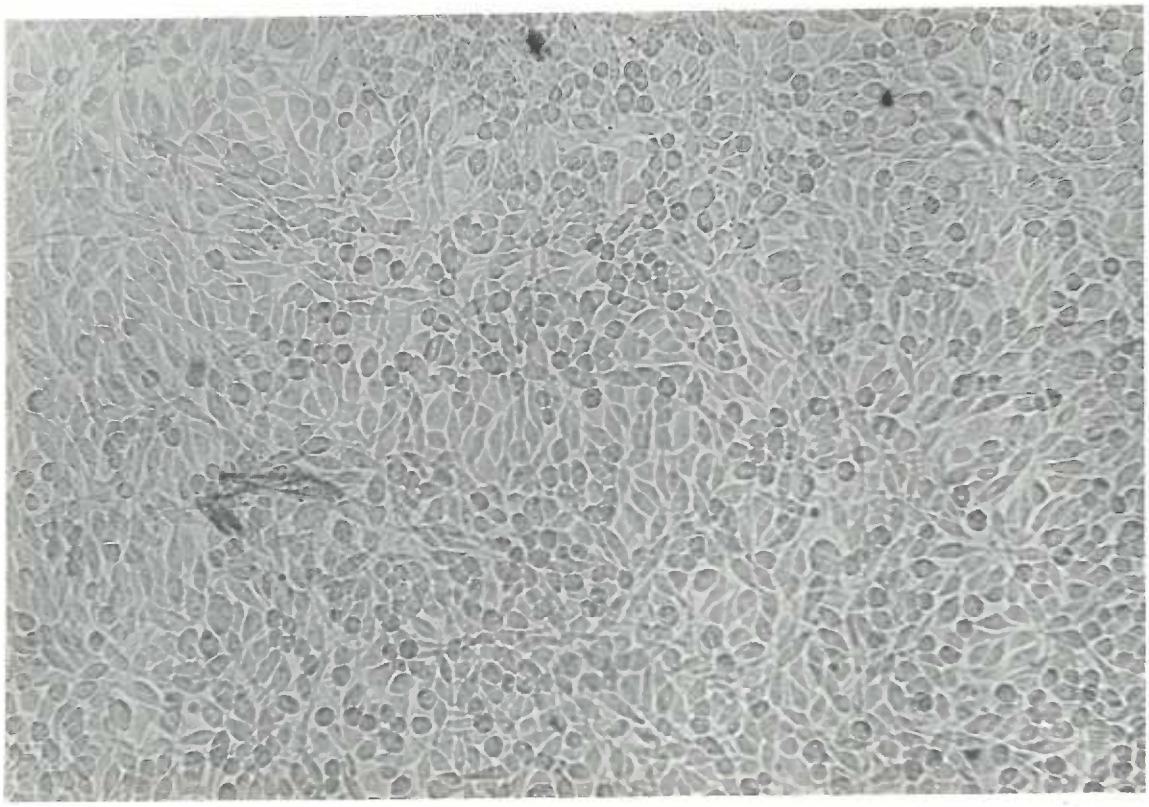


Plate 3

Virus-induced cytopathology in L-929 cell monolayer 48 hr after inoculation with Colorado tick fever virus at a virus:cell ratio of approximately 1:50. (950X)

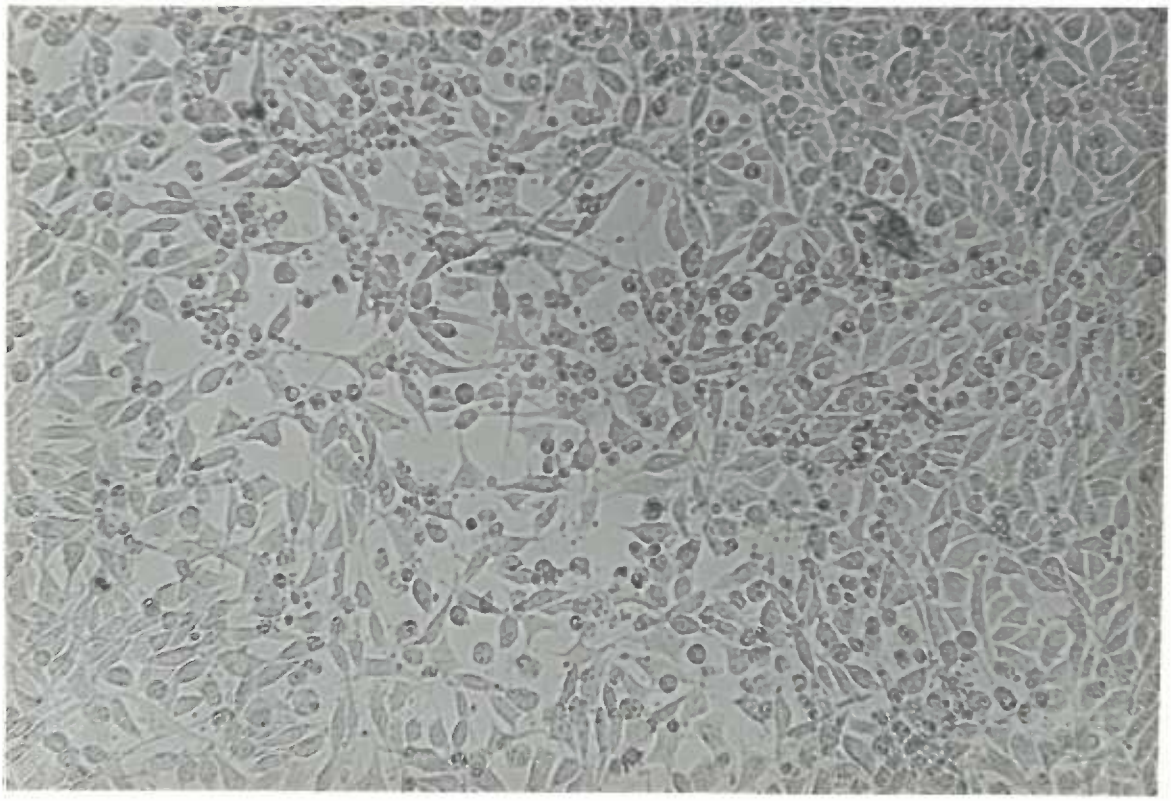


Plate 4

Virus-induced cytopathology in L-929 cell monolayer 72 hr after inoculation with Colorado tick fever virus at a virus:cell ratio of approximately 1:50. (950X)

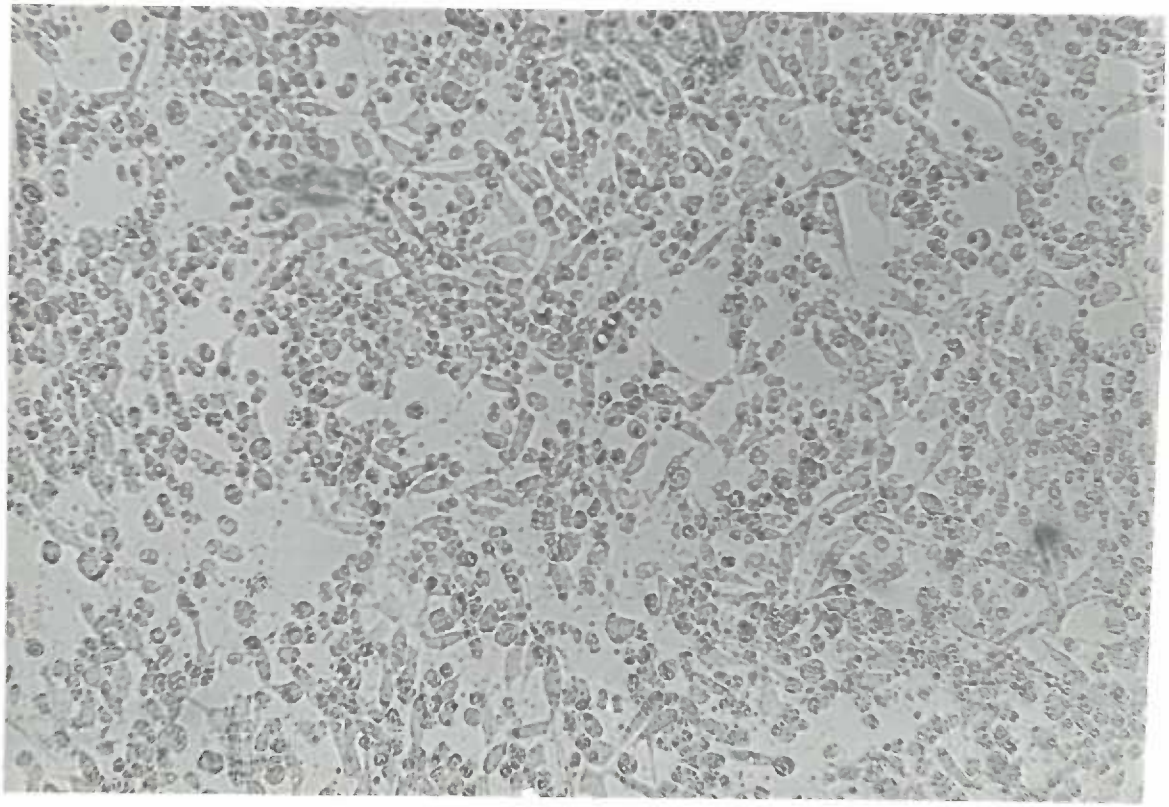


Plate 5

Electron micrograph of a "virus-like particle" approximately 50 mμ in diameter; abundant in Colorado tick fever virus suspensions diluted in 1 M potassium citrate. Shadow-cast at 17 deg from horizontal with platinum. Instrument magnification: 50,000 X; total magnification: 200,000 X.

Plate 6

Electron micrograph of a "virus-like particle" approximately 50 mμ in diameter; abundant in Colorado tick fever virus suspensions diluted in 1 M potassium citrate. Shadow-cast at 17 deg from horizontal with platinum. Instrument magnification: 50,000 X; total magnification: 400,000 X.

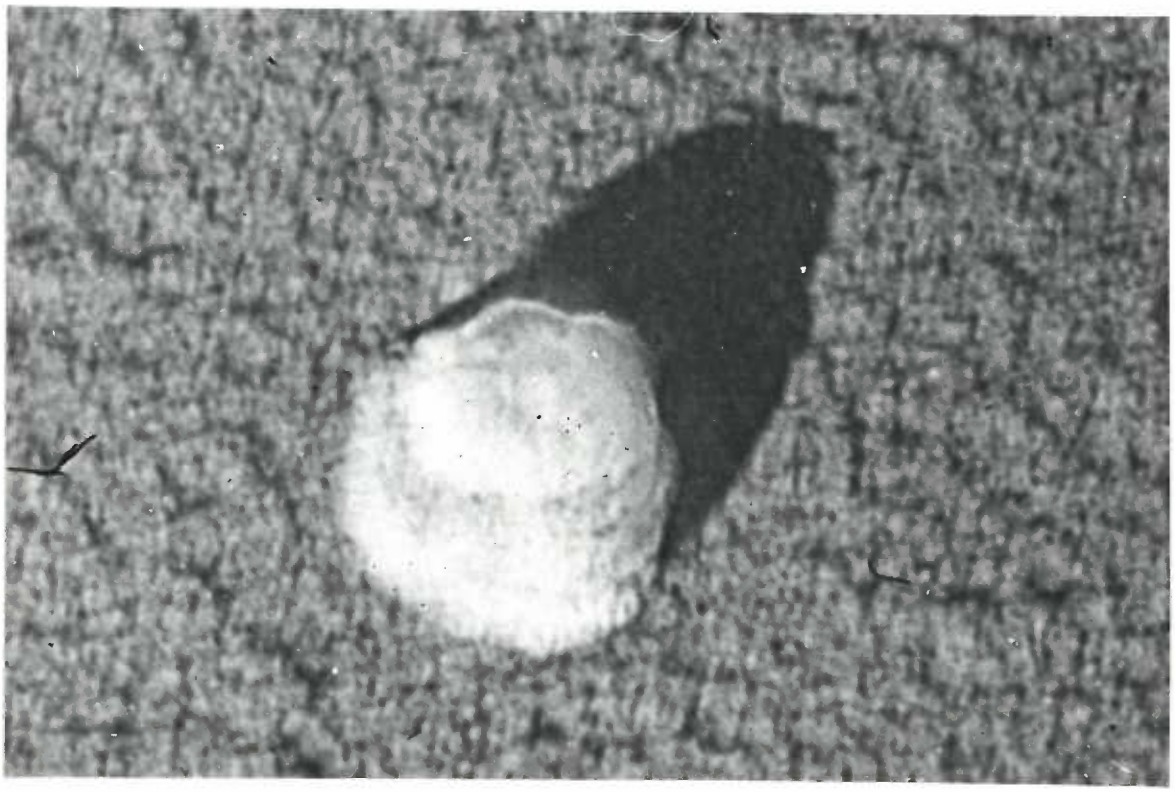
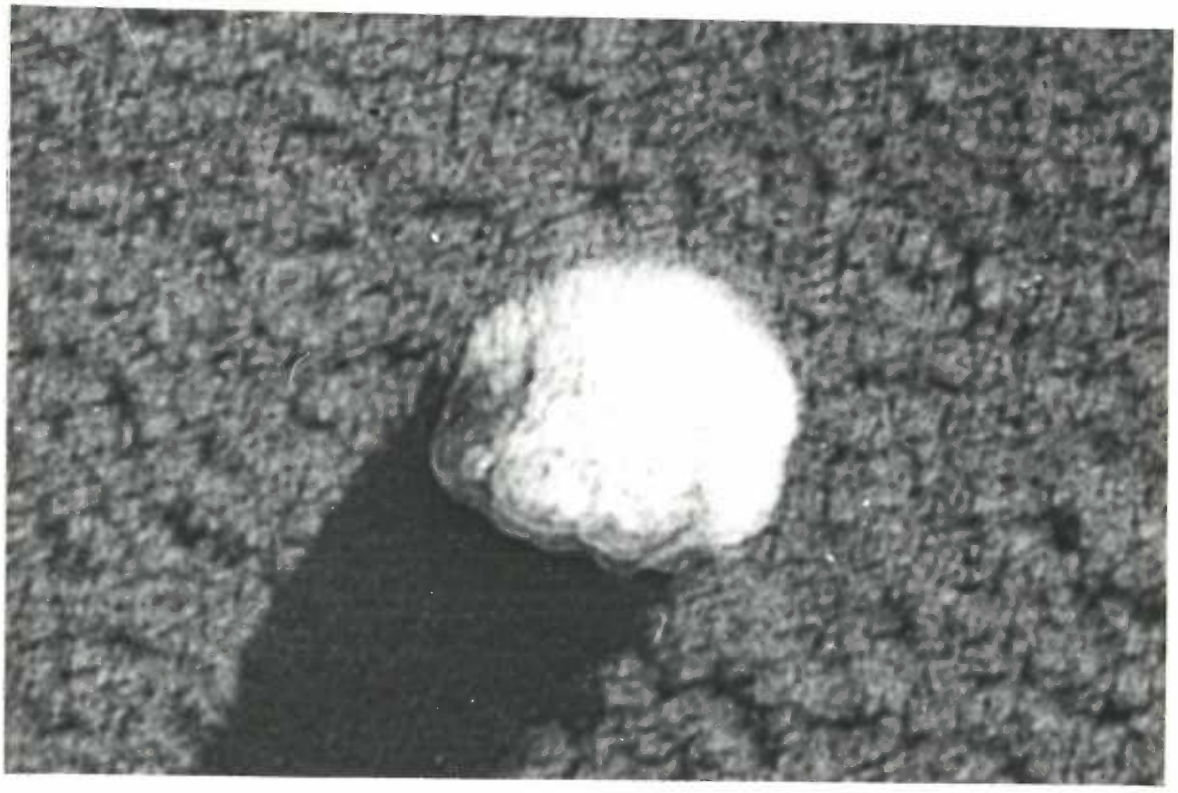


Plate 7

Electron micrograph of a "virus-like particle" approximately 50 mμ in diameter; abundant in Colorado tick fever virus suspensions diluted in 1 M potassium citrate. Shadow-cast at 17 deg from horizontal with platinum. Instrument magnification: 50,000 X; total magnification: 400,000 X.



CONCLUSIONS

Infected mouse brain homogenate preparations of Colorado tick fever virus, strain GS-20, are inactivated at 50 C in a classic two-component fashion. Both the initial "fast" and subsequent "slower" components are linear when expressed as semi-logarithmic functions, indicating that both obey first-order (i.e., single reacting species) kinetics. The precise slope of each component appears to be dependent upon the particular virus pool, while within each pool the decay kinetics are readily reproducible. Inactivation of viral aliquots at 37 C yields only a single evident component.

Sodium and potassium citrate were found to stabilize CTFV preparations in the presence of 10% (v/v) normal rabbit serum (NRS) at 50 C more efficiently than NRS alone. All other diluents tested, however, enhanced the rate of decay of the virus at 50 C.

The estimated half-life of CTFV at 50 C (1% infected mouse-brain homogenate in NRS) is approximately 3 min, while at 37 C the calculated half-life of the same virus pool is about 10 hr. Inserting the derived velocity constants (k_1, k_2) for inactivation of the virus at these two temperatures into the Eyring equation gives an estimate of the heat of activation (ΔH) and the entropy of activation (ΔS) for the thermal decay event. Both ΔH and ΔS were found to be relatively large positive values, indicating a reaction which is markedly temperature-dependent and yields a product significantly more random (less orderly structured) in configuration. Since only deoxyribonucleic acid (DNA) and proteins are characterized by such kinetics (and CTFV is known to lack essential DNA), the logical assumption is that CTFV is inactivated at 50 C by a change in the protein component of the virus.

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