Virus-Like Sensitivity of the Scrapie Agent to Heat Inactivation

Abstract. *The resistance of the infectious agent of scrapie disease to sterilization at 100° or 121°C is reputed to be inconsistent with the structure of conventional viruses. However, in kinetic studies the majority of hamster scrapie strain 263K infectivity was (like that of previously characterized viruses) rapidly inactivated at temperatures of 100°C or greater. Small resistant subpopulations remained. Similar heat-resistant subpopulations were observed at 60°C for phage λ but only in the presence of brain homogenate. Brain homogenate may also confer stability to small subfractions of scrapie infectivity. Such refractory subpopulations cannot be used to make structural inferences that are properly obtained from the behavior of the majority population as revealed in the initial inactivation.*

The infective agent of scrapie disease, a lethal slow infection of the central nervous systems of sheep and goats, is resistant to sterilization by many common inactivants of viruses (*1–3*). Some investigators have interpreted this resistance as indicating an agent with unique physical properties (*4*). In particular, scrapie's resistance to sterilization by boiling or autoclaving has seemed to exclude conventional virus structures (*1, 2, 5–9*). However, sterilization requires total destruction of infectivity and therefore measures only the most resistant subpopulation of a virus. In contrast, structural information is more properly deduced from the behavior of the majority population of a virus as revealed in the initial inactivation. Past investigations of scrapie's sensitivity to 100° or 121°C have established that, while scrapie is incompletely inactivated by exposure to either temperature for 1 hour, there is significant loss of infectivity. In the few instances where inferences can be drawn about the kinetics of this inactivation (*6, 7*), scrapie's thermal behavior could be interpreted as rather conventional, although the experiments have usually been interpreted according to the prevailing paradigm of scrapie's unusual stability. In the study reported here, the thermal stability of a laboratory strain of scrapie, hamster scrapie strain 263K (*10*), was measured in carefully controlled kinetic experiments at 60°, 80°, 100°, and 121°C. Scrapie infectivity was found to be sensitive to temperatures of 100°C or greater in a manner consistent with the behavior of conventional viruses.

Hamster brain was homogenized by sonication, sealed into serum bottles, and brought rapidly to the test temperatures by means of a thermal pulse (*11*). A thermistor sealed into one of the bottles was used to obtain a complete temperature record of each experiment (Fig. 1). Control experiments were performed on bacteriophages fd, λ, and PM2, suspended either in normal brain homogenate or phosphate-buffered physiological saline (PBS) at *p*H 7.2. Each sample was rehomogenized after thermal exposure and the scrapie infectivity assayed by end-point dilution in golden Syrian hamsters (Fig. 2).

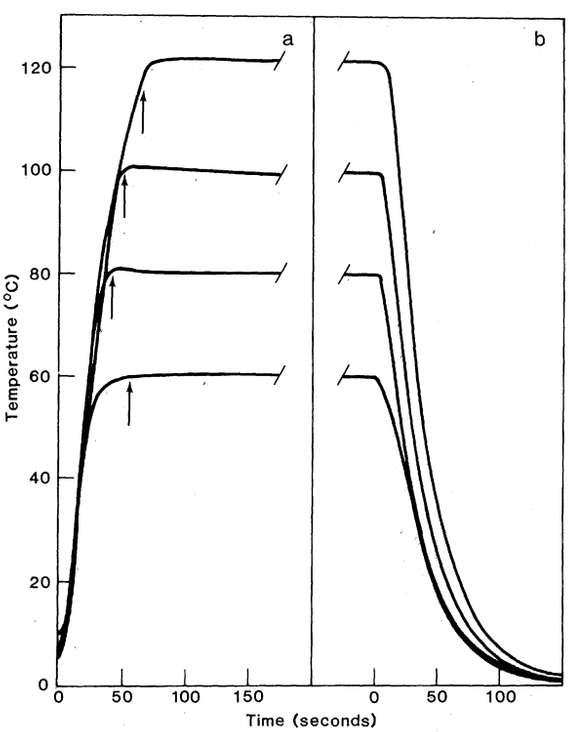

Fig. 1. Temperature profiles of scrapie inactivation. The thermal equilibration of each group of samples was monitored by continuous recording from a thermistor (*22*) placed at the center of fluid mass of one of the samples. Data presented are for the scrapie inactivations in Fig. 2. (a) Heating. Arrows indicate the points at which the time zero samples were taken. (b) Cooling. Data are for the last sample taken in each group. Temperatures were constant to within ±1°C of the test temperature in the time interval between (a) and (b).

When scrapie strain 263K was exposed to 121°C, 99.9999 percent of the infectivity was destroyed during the minute required to bring the sample to temperature (Fig. 2). At 100°C, 97 percent was destroyed within 2 minutes of exposure at temperature (Fig. 2). Thus the majority of scrapie infectivity is inactivated by brief exposure to temperatures of 100°C or greater. At both temperatures small subpopulations of the infectivity survived for longer periods. Resistant subpopulations are a common observation in virology (*12, 13*) and they pose important medical, agricultural, and public health problems in the areas of decontamination, disinfection, and vaccine production (*12, 13*). As a consequence, there is great interest in the mechanisms by which small subpopulations such as these obtain resistance. Nevertheless, the behavior of subpopulations has little bearing on the structural characteristics of the virus. For structural information one must look to the behavior of the majority population that is revealed in the initial inactivation process. In the case of the scrapie virus, the insensitivity of the infectivity assay makes it difficult to observe the initial inactivation unless it is dramatic. When scrapie was exposed to 100° or 121°C the loss of scrapie infectivity was sufficiently great and sufficiently abrupt to reveal a normal viral sensitivity to these temperatures.

Scrapie's sensitivity to 60° or 80°C (Fig. 2) is uncertain. The end-point dilution assay for infectivity which has been used in this and previous determinations of scrapie's thermal sensitivity cannot, without the expenditure of impracticably large numbers of animals, detect less than a 70 percent loss in infectivity (approximately $10^{0.5}$ surviving fraction) from one point to the next in an inactivation experiment (*9, 14*). Because of this inability to distinguish complete insensitivity from initial inactivation of up to 70 percent of the infective population, negative experiments say little about the structure of the scrapie agent.

The informational content of a low-level inactivation can be improved somewhat by observing the trend of the data in a multipoint kinetic experiment. Nevertheless, it is not possible on the basis of the 60° and 80°C experiments in Fig. 2 to conclude anything about the sensitivity of the majority population of the scrapie infectivity to these temperatures. One can only say that, if inactivation

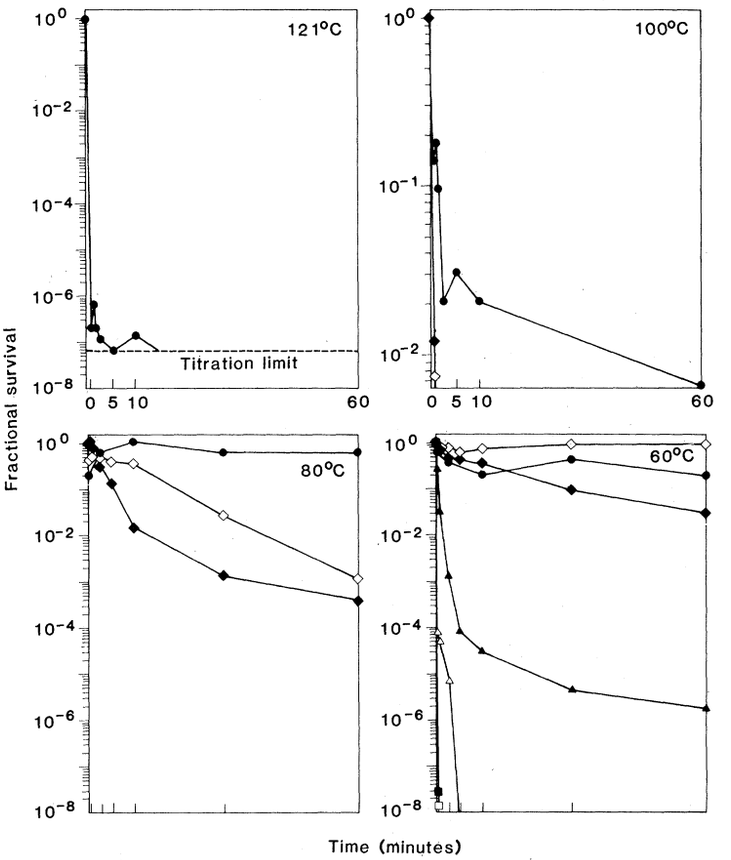

Fig. 2. Inactivation of scrapie and three other viruses by heat. Brains removed from CO_2-asphyxiated hamsters in early clinical disease with scrapie strain 263K (*10*) were sonicated in 10 percent suspension in PBS for 6 minutes at full power with a Braunsonic 1510 and standard probe. Uninfected brains were used for the bacteriophage controls. In each experiment eight 7.5-ml portions of either the scrapie suspension or the control viruses were sealed into 10-ml serum bottles (which were arranged in a radial pattern in special holders to ensure identical exposure), immersed in accurately regulated oil baths (*11*) (Fig. 1), and removed with agitation at timed intervals to a 0°C bath. A recording thermometer was used to obtain a complete temperature record of each experiment (Fig. 1). The time zero sample was removed as soon as the recording thermometer indicated that the samples had reached the test temperature. Subsequent samplings were timed from this point which is indicated by the arrows in Fig. 1. In each experiment one bottle was held at 4°C as the unexposed control. To counter the effects of aggregation (*23*) we subjected each scrapie sample to violent homogenization in its test bottle using 25 seconds of pulsed sonication from a Braunsonic 1510 sonicator equipped with the intermediate probe and operated at 200 watts (*24*). The phage experiments required gentler methods of homogenization to prevent inactivation of phages fd or λ (*19*). The order of homogenization was from the most inactivated (longest time point) to the least inactivated (shortest time point) sample with autoclave sterilization of all tools between each experiment. The freshly sonicated samples were serially diluted through tenfold steps with vortex mixing after each step, transferred to a serum bottle, capped, snap-frozen in a Dry Ice–methanol bath, and stored at −70°C for 3 days. At each dilution four weanling female outbred golden Syrian hamsters (Charles River–Lakeview Hamstery, Lakeville, N.J.) were inoculated intracerebrally in the left hemispheres with 0.05-ml portions of the freshly thawed and vigorously vortex-homogenized sample. The animals were observed for 310 days and scored positive for scrapie if they developed obvious clinical disease (*25*) or, in doubtful cases, if they showed, among a group of coded specimens, characteristic spongiform pathology upon histopathological examination of the brain (*26*). The titration end points were calculated by the method of Reed and Muench (*27*) and virus survivals computed from the average value of the 4°C controls. Symbols: ●, scrapie; ▲, △, phage λ; ◆, ◇, phage fd; and ■, □, phage PM2. Solid symbols represent brain homogenate; open symbols represent PBS.

occurs, a subpopulation of 10 percent or more of the virus is resistant to these temperatures.

Even complete resistance to these relatively high temperatures would not constitute a unique property of the scrapie agent. Highly purified phage fd is stable at 60°C for at least 2 hours (Fig. 2) and only slowly inactivated at 80°C (Fig. 2). Aleutian disease virus, a parvovirus, is similarly stable, especially in the presence of mink brain homogenate (*15*). Many viruses are resistant to 50°C (*13, 16*) and may be resistant to even higher temperatures as well.

Viral stability at 80°C seems less remarkable in view of the recent discoveries of a thermophilic microbe with a growth optimum at 105°C (*17*) and submarine hydrothermal vent organisms that can grow at 250°C and be isolated from 306°C water (*18*). That these organisms display not only structural stability but also metabolic activity at high temperatures warns against overinterpretation of circumstantial inactivation data in drawing structural conclusions about the scrapie agent.

This study and past determinations of scrapie's thermal stability were conducted in the presence of brain homogenate. The effect of 10 percent normal hamster brain homogenate on thermal inactivation varies with the virus tested. Phage λ is much more stable in the presence of brain than in purified form. A small subpopulation, 0.005 percent of the total λ, is highly resistant to 60°C inactivation in the presence of brain homogenate, as revealed by the plateau in the λ inactivation curve after 10 minutes (Fig. 2). Phage fd is more sensitive at all temperatures tested when in the presence of brain homogenate (*19*). Phage PM2, a lipid-containing phage, was almost instantly inactivated at all four temperatures regardless of the presence or absence of brain. Apparently brain homogenate itself confers a measure of thermal stability to some viruses and especially to small subpopulations. Thus the resistant scrapie subpopulation seen at 100°C, like that of λ at 60°C, may be due more to the molecular environment of the virus than to an intrinsic property of the virus itself. The stabilizing effect of brain homogenate on the chemical inactivation of these same test viruses is also pronounced (*14, 20*).

The resistance of small subpopulations of scrapie infectivity to inactivation at 100°C dictates against the use of boiling for disinfection of scrapie-contaminated material. Resistant subpopulations can also exist at 121°C (*1, 2, 6, 8, 9*). In other experiments we have recovered small amounts of infectivity from a sample exposed to 121°C for 60 minutes in an autoclave (*1*). The surviving population represented only 0.000002 percent of the starting infectivity. Nevertheless, this result suggests caution in the use of autoclaves for disinfection of scrapie and other members of this class of agents (*1, 3, 21*).

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11. Thermal inactivation experiments are complicated by the asymptotic approach to thermal equilibrium. In these experiments the equilibration period was minimized and all samples were brought to the test temperature in 60 seconds or less by means of a brief thermal pulse in a 156°C oil bath. The temperature of the vigorously agitated samples was monitored with a recording thermometer (Fig. 1) and the samples were transferred at an empirically determined point to the test bath in such a way that the temperature continued to rise quickly to the test temperature and then stopped.
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19. Phage fd was inactivated by the violent homogenization procedure applied to the scrapie samples. Therefore, phage fd–containing samples had to be homogenized by gentler non-inactivating means 10 seconds at 70 percent of full power with a Tekmar SDT Tissumizer (Tekmar Co., Cincinnati, OH). If phage fd aggregates with itself or with tissue coagulated during exposure to heat, the lower relative titers seen for phage fd in 10 percent brain homogenate may reflect inadequate homogenization of this virus.
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21. In thermistor-controlled tests of our own autoclaves we have observed long delays in reaching 121°C, depending on the mass and type of sample. Some surfaces of the glassware placed in a kill pan full of empty glassware can take over 1 hour to reach 121°C in an ambient environment of 121°C (*14*). This suggests that some of the survival after autoclaving may be due to incomplete exposure to 121°C. Because the approach to thermal equilibrium is asymptotic, 121°C is attained much more readily by operating the autoclave at 132°C (*14*). Therefore, it may be advisable to operate the autoclave at that temperature to ensure that all materials contaminated with scrapie or other subacute spongiform virus encephalopathies spend as much time as possible at 121°C or greater.
22. The output of Yellow Springs Instruments No. 421 thermistor was monitored by a Yellow Springs Instruments Model 42 Tele Thermometer and recorder. The measured time constant of the thermistor configured in this way was 5.3 seconds.
23. When exposed to 100° or 121°C, brain homogenate coagulates into soft gray curds. At 80°C the homogenate gradually turns gray and grainy over an hour's time. At 60°C samples remain homogeneous and retain their pinkish color for almost an hour.
24. Previous tests have shown scrapie infectivity titers to be stable to this treatment (R. G. Rohwer, unpublished data).
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28. I thank G. Sokol for technical assistance, E. Green for supervision of the animal colony, and D. C. Gajdusek for support, encouragement, and critical reading of the manuscript.

23 June 1983; accepted 19 December 1983

A New Type D Retrovirus Isolated from Macaques with an Immunodeficiency Syndrome

Abstract. *Macaque monkeys with the recently described acquired immunodeficiency syndrome show a marked defect in T-lymphocyte function and die with opportunistic infections and lymphoproliferative abnormalities. In the study described here a new type D retrovirus was isolated from two* Macaca cyclopis *with this syndrome. This virus is related to, but distinct from, Mason-Pfizer monkey virus, a type D retrovirus previously isolated from a mammary tumor of a rhesus monkey* (Macaca mulatta).

Endemic immunodeficiency diseases in colonies of macaque monkeys at the New England Regional Primate Research Center (NERPRC) and the California Primate Research Center at Davis have many similarities to the acquired immunodeficiency syndrome (AIDS) in humans (*1*, *2*). Consistent immunologic abnormalities have been noted in affected macaques of the NERPRC colony. Proliferative responses of their peripheral blood lymphocytes to antigens and lectins are dramatically diminished (*1*). Furthermore, ratios of the T4 (helper/inducer) to T8 (suppressor/cytotoxic) circulating T-lymphocytes of the macaques in the colony at greatest risk for developing this syndrome (*Macaca cyclopis*) are considerably less than those in other macaques (*1*). Peripheral blood smears from affected monkeys reveal an immature circulating mononuclear cell with vacuolated cytoplasm and prominent nucleoli (*1*).

We have transmitted spontaneously occurring lymphomas in rhesus monkeys to healthy monkeys by means of tumor cell suspensions (*3*). The recipient animals developed undifferentiated or poorly differentiated lymphomas or parenchymal lymphoproliferative abnormalities suggestive of early lesions of lymphoma. They also developed opportunistic infections with agents such as cytomegalovirus (CMV) and *Cryptosporidium* and showed evidence of an abnormal peripheral blood mononuclear cell morphologically similar to that seen in macaques with the immunodeficiency syndrome. These findings suggested a link between the transmissible lymphomas and the immunodeficiency syndrome in macaques. In fact, by inoculating previously healthy macaques with either tissue or a cell-free filtrate from a macaque lymphoma we were able to transmit the immunodeficiency syndrome (*4*). The recipients developed evidence of profound lymphocyte dysfunction or died with infections by opportunistic agents, including *Candida albicans*, *Cryptosporidium*, and CMV.

These studies implicated an infectious agent in the syndrome. Attempts to isolate a virus from macaques with this syndrome were made by using explant cultures of minced tissues (lymph nodes, spleens, and parenchymal tissues) and by cocultivation of cell suspensions, minced tissues, and throat and rectal swabs with a battery of indicator cell lines. Viruses identified by these approaches included adenoviruses, simian virus 40 (SV40), CMV, paramyxoviruses including measles virus, and foamy viruses. Viruses were identified on the basis of reactivity in indirect immunofluorescence tests with specific antisera and by electron microscopy. None of these viruses, however, seemed likely to be etiologic in the macaque immunodeficiency syndrome.

We reasoned that since the presumed etiologic virus was causing immunosuppression, we might be able to isolate it by cocultivation of tissue from an infected animal with lymphoid cell lines. We used tissue from two 3-year-old *M. cyclopis* (Mc) in late stages of the immunodeficiency disease. One, Mc 184-80, was dehydrated and had lymphadenopathy (*5*). Peripheral blood mononuclear cells (PBM) from this animal showed no significant in vitro proliferative responses to pokeweed mitogen, concanavalin A, xenogeneic cells, or *Candida albicans* antigen. Lymph node biopsies 1 month