Thermal Inactivation of Poliovirus in the Presence of Selective Organic Molecules (Cholesterol, Lecithin, Collagen, and β-Carotene)

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Poliovirus type 1 strain LS-a exhibited the typical thermal inactivation pattern observed previously by other investigators for poliovirus strains sensitive to the temperatures used in these experiments. However, when the virus suspension was thermally treated at 121 C for 5 sec in the presence of 2% collagen, a stabilizing effect on the virus was observed. The stabilizing effect in the presence of other food additives, such as cholesterol, lecithin, or β-carotene, was less dramatic or there was no effect at all. Pretreatment of the cells with the same additives before inoculation induced various changes in the susceptibility of the cells to infection by poliovirus. Lecithin and cholesterol treatment appeared to increase HeLa cell susceptibility to the invading virus, thereby enhancing infectivity. Ultraviolet examination of thermally inactivated virus (121 C) suspensions did not indicate any severe denaturation of the nucleic acid core. Subsequent phenol extraction of the infectious nucleic acid from the heat-inactivated virions revealed that infectious nucleic acid was still present in the denatured heat-treated (62 to 72 C) samples of virion. The immediate past history of treatment of the uninoculated cells appeared to be important, since pretreatment of the cells with cholesterol before inoculation resulted in a noticeable increase in infectivity. In addition, cholesterol-treated uninoculated cell sheets also exhibited an increase in longevity compared to the uninoculated, untreated controls.

The observation that a wide variety of viruses may be transmitted by foods handled by infected individuals (3) suggests that physical or chemical treatment may be necessary after handling but before distribution of the foods. Treatment at this time would insure that the medium would not contain infectious virus; however, the difficulty of detecting the virus in the food because of relatively insensitive assay methods could pose a problem (2, 11, 12). It is known that some enteroviruses, such as poliovirus, occur as heat-resistant and heat-sensitive forms (5, 16). With these considerations in mind, it was decided to use a defined system in which the quality and quantity of food additives and the assay method could be controlled. In addition, the selection of temperatures for the treatment of these heterogeneous systems was based upon the known fact that the thermal processes designed to destroy viable fungal and bacterial spores do not necessarily insure the destruction of viruses entrapped in foods (10). For example, it has been shown that enteroviruses entrained in milk are not completely inactivated at 55 C for the length of the time of pasteurization (3). Therefore, treatment of foods by heat for a definite period of time does not insure complete inactivation of infectious particles from a heterogeneous or homogeneous population.

Poliovirus was selected for this study because of its availability, its assayability, and the abundant information on its inactivation kinetics. In this study, I investigated (i) the effects of different food additives on the infectivity of poliovirus suspensions thermally treated at 121 C, (ii) the effects of pretreatment of the cells with various food additives on virus infectivity, and (iii) whether elimination of virus infectivity inactivated the viral genome.

MATERIALS AND METHODS

Preparation of confluent monolayers. HeLa P cells (human carcinoma) were cultured in 60-cm² prescription bottles overlaid with Hanks balanced salt solution (pH 6.9) containing 5% fetal calf serum. The cultures were planted at a cell density of 10⁶ cells per ml, gassed with sterile 5% carbon dioxide, and in-
The virus was propagated in HeLa P cells grown in a suspension medium at pH 6.9 for 24 hr.

The prior history of the HeLa cells used for the suspension cultures is as follows. The cells were grown as monolayers as described above; upon confluency of the monolayer, the cultures were washed with two samples of complete suspension cells and in turn were grown in suspension in minimal essential medium (MEM) plus glutamine, 10% fetal calf serum, and 0.6 ml of a 0.3% suspension of sterile Methocel (15 centipoise) at a cell density of 10^6 cells/ml for 200 ml. These suspension cultures were perfused slowly with a 5% sterile carbon dioxide-air mixture at a gas to liquid ratio of 4 to 1 for 5 min. At this time, the suspension cultures were capped and agitated at 100 rev/min on a New Brunswick (model G 25) gyratory shaker-incubator at 37 C. In approximately 2 days, these cells were split back to 10^6 cells/ml from 8 × 10^6 cells/ml, and fresh media were added. At this point, the cells were recovered by centrifugation at 250 × g.

The precipitated cells were washed with incomplete MEM media minus the fetal calf serum and centrifuged at 250 × g for 5 min. The precipitated cells were then suspended in incomplete MEM media, infected with the poliovirus at a multiplicity of infection (MOI) of 20, and incubated further at 110 rev/min for 24 hr. A 50-ml sample of the infected cell suspension was centrifuged, in the manner described above, resuspended in 5 ml of phosphate-buffered saline (PBS; pH 7.0), and frozen at -19 C for 4 hr. The infected cells were thawed, the cell debris was removed by centrifugation in the cold at 12,100 × g, and the virus from both supernatants was recovered by a simplified procedure outlined by Levintow and Darnell (13). The virus purification was only completed to the concentration step. Titration of the virus was done on HeLa P cells, and the titer was 3.0 × 10^8 plaque-forming units (PFU) per ml. This virus suspension was subdivided into 1-ml samples and frozen and stored in liquid nitrogen until used. A sample was examined by ultraviolet, and the weight extinction coefficient (E_1%_1cm = 81.6; reference 1) was used to quantify the amount (micrograms per milliliter) of virus. A value of 0.076 absorbance units at 260 nm gave us a 2.7-mg yield of virus. This virus preparation at 3.0 × 10^8 PFU per ml was used for the thermal inactivation studies.

Plaquing Procedure. Confluent monolayers of HeLa P cells were rinsed twice with sterile PBS (pH 7.0) to remove any residual serum. The plaquing medium was composed of 57.6 ml of Earle’s salts, 1.6 ml of penicillin-streptomycin (10,000 units per ml), 1.6 ml of glutamine [200 mM, 1.6 ml of essential amino acid mixture (100×)], and 1.6 ml of complete vitamins (Grand Island Biological Co.) This mixture was filter-sterilized, added (v/v) to 3% sterile Difco agar, subdivided into 9.8-ml samples, and stored in the refrigerator at 12 C until used. The fetal calf serum was preheated at 55 C for 10 min, and 0.2-ml samples were added to each monolayer as needed.

The cells were inoculated with 0.5 ml of the virus, and the suspension was diluted accordingly, with 10-fold dilutions to 10^−1. The virus was allowed to adsorb at 8 C for 60 min, and the infected cells were washed with PBS (pH 7.0). These infected cultures were then incubated at 36 C for 1 hr, and the plaquing was performed using (45 C) plus 0.2 ml of sterile, heat-inactivated fetal calf serum were overlaid over the infected monolayer. The agar was allowed to solidify, and the cultures were incubated at 37 C until the plaques were visible; this was usually about 47 hr as seen under a 10× light microscope, with the plaques being visible in 72 to 96 hr. At 72 hr the cultures were overlaid with 0.5 ml of 0.2% neutral red solution and incubated at 12 C for 2 hr; the plaques were then counted under a Quebec colony counter.

Preparation of sterile β-carotene, collagen, lecithin, and cholesterol. These compounds were suspended individually in sterile glass-distilled water, 0.4 g/ml, or 0.2 g/ml, and the suspensions were autoclaved at 15 psi and 121 C for 10 min. During the interim before use in the virus experiments, the suspensions were refrigerated at 6 to 8 C. The virus suspensions were mixed with suspension containing the organic compounds as described elsewhere for the inactivation experiments and treatment of the cells.

Heat inactivation experiments. Virus preparations were heat-inactivated in a manner described by Stern and Proctor (17). The use of such a technique enabled us to minimize the time that would elapse while the heat-treated virus sample was being transferred between the hot oil and the ice-water bath. The average time for this exchange was 0.38 ± 0.02 sec (Andersen, personal communication). The sterile capillary tubes (1.2 to 1.5 mm in diameter by 75 mm in length; 40-μliter capacity) contained 20 μliters of the virus suspension. The use of these tubes minimized the thermal gradient in the oil and ice-water baths and facilitated the handling and transfer at the conclusion of the thermal treatment.

The 40-μlter capillary tubes were filled with 20 μliters of the virus suspension and sealed at both ends. During the loading, the tubes were kept in the cold to minimize the thermal effects from the surroundings. These sealed tubes were then immediately transferred to an aluminum tray containing 10 holes large enough to hold the capillary tubes. This loaded aluminum tray was submerged into an oil bath for the thermal heat treatment at a predesignated temperature. At the end of the thermal treatment, a clock mechanism tripped an electromagnet releasing the aluminum tray-sealed vials into cold water. The vials were removed and one end was cut off; the liquid was withdrawn and diluted with 10 ml of sterile PBS.
(pH 6.9). A 0.5-ml amount of this suspension was then added to the confluent HeLa monolayers and assayed as previously described.

**RESULTS**

**Thermal inactivation studies.** In the preliminary inactivation experiments at 62 and 72°C (Table 1), heating for 40 min did not completely destroy the infectivity, but with heating at 72°C for 60 min the infectivity was completely inactivated. At 80°C for 15 min and also at 121°C for 20 sec, infectivity was completely inactivated. These results indicate that the greatest percentage of virus particles were inactivated following first-order kinetics within 10 min. However, after the reaction had proceeded for a short period of time, the rate of loss of infectivity was less dramatic. This change in the rate of the loss suggests that a mixed population of heat-resistant and heat-labile virus was involved or that the inactivated virus particles were protecting the residual infectious virus particles from the thermal effects.

**Ultraviolet examination of heat-treated virus suspension.** Virus suspension heated at 121°C and examined by ultraviolet from 220 to 360 nm did not show any dramatic change in the spectrum; however, there were subtle changes in the spectrum in the ξ_{max} area (260 nm). A slight hyperchromic shift at this ξ_{max} of 0.07 to 0.1 absorbance unit was observed with very little change in other parts of the spectrum. There was no obvious hypochromic or bathochromic shift in the spectrum of the ξ_{max} or the total spectrum. Therefore, this slight delta shift in the ξ_{max} would suggest that the integrity of the viral ribonucleic acid (RNA) was still intact and that the observed change in optical density resulted from denaturation of the protein coat and its effect on the nucleic acid spectrum. No attempt was made at this time to recover naked infectious RNA from the virus suspension that was treated at 121°C. Twenty seconds after thermal treatment of another virus suspension at 121°C, a sample was again examined by ultraviolet from 220 to 360 nm; at that time a 15% change in the absorbance characteristics at 260 nm was observed, although there was no significant change in the spectrum elsewhere between 220 and 360 nm, as discussed above for the 5-sec heat-treated sample.

**Examination of RNA.** The viral RNA recovered from a viral suspension sample, heated at 121°C for 20 sec and partitioned with an 80% phenol solution at 20°C, revealed no residual infectivity. The RNA extracted from viral suspensions heated at 62 and 72°C for 60 min yielded 12 to 16 plaques and 4 to 10 plaques, respectively, indicating that the loss in infectivity of the heat-treated virus could result primarily from the denaturation of the virus capsid protein and that the initial rapid inactivation rate could result from destruction of the viral attachment sites.

**Effect of added organic molecules on thermal inactivation of virus at 121°C.** Cholesterol, lecithin, collagen, and β-carotene were selected for study of their stabilizing effects on the virus during thermal treatment at 121°C. These selections were based upon their prevalence in many foods which are thermally processed.

The heating of the virus suspension at 121°C for 5 sec was preselected for the thermal treat-

**Table 1. Thermal inactivation kinetics of poliovirus**

| Temp (°C) | Time (min) | Residual PFU/ml |
|-----------|------------|-----------------|
|           |            | Expt 1          | Expt 2          |
| 62        | 0          | 3.0 × 10^8      | 3.0 × 10^8      |
|           | 10         | 98 ± 3          | 93 ± 3          |
|           | 20         | 34 ± 6          | 33 ± 3          |
|           | 40         | 17 ± 5          | 14 ± 2          |
|           | 60         | 5 ± 2           | 4 ± 2           |
| 72        | 0          | 3.0 × 10^8      | 3.0 × 10^8      |
|           | 10         | 86 ± 1          | 86 ± 3          |
|           | 20         | 32 ± 6          | 38 ± 4          |
|           | 40         | 15 ± 2          | 12 ± 2          |
|           | 60         | 0               | 0               |
| 121       | 0*         | 3.0 × 10^8      | 3.0 × 10^8      |
|           | 5          | 205 ± 6         | 206 ± 6         |
|           | 10         | 117 ± 2         | 120 ± 4         |
|           | 15         | 31 ± 2          | 44 ± 12         |
|           | 15*        | 0               | 0               |

* Time periods for a temperature of 121°C were measured in seconds.

| Expt | Compound  | Residual PFU/culture |
|------|-----------|----------------------|
| 1    | Collagen  | 926 ± 69             |
|      | Lecithin  | 23 ± 5               |
|      | β-Carotene| 64 ± 9               |
|      | Cholesterol| 25 ± 3              |
|      | Control   | 22 ± 4               |
| 2    | Collagen  | 916 ± 41             |
|      | Lecithin  | 25 ± 6               |
|      | β-Carotene| 48 ± 12              |
|      | Cholesterol| 23 ± 2              |
|      | Control   | 24 ± 4               |

* Diluted 10 times for plaquing. Original titer, 3.0 × 10^8 PFU/ml.
ment of the suspension. The virus suspension heated in this manner gave approximately 200 PFU per culture when assayed (Table 2) and required a 10-fold dilution for quantitation.

Table 2 shows that 2% collagen stabilized the virus against the inactivating effects of the thermal energy, but to a lesser degree as did β-carotene. Cholesterol and lecithin did not stabilize the virus. These compounds had no apparent enhancing effect on virus infectivity after thermal treatment. However, cholesterol did increase the infectivity (Table 3) when the cells were incubated in its presence before exposure to the virus. The infectivity increased 130 to 150% above the controls. Lecithin-treated cells, before inoculation with virus, exhibited an increase in infectivity by 90 to 110% of the controls. The collagen-pre-treated (inoculated) cells exhibited a 25% increase in infectivity, whereas β-carotene-pre-treated cells exhibited a slight inhibition in infectivity. In each case, the cells pretreated with the different compounds did exhibit a differential response. If the cells pretreated with the compounds were washed with fresh media, the cells remained healthy. The pretreated inoculated cells that were not washed before overlaying with agar died. The confluent cell sheet treated with 2% cholesterol or lecithin overlay with agar exhibited unusual morphological responses within 48 hr after treatment in that they appeared distended but healthy, whereas the cells treated with collagen or β-carotene appeared normal and healthy. At 80 to 96 hr after viral inoculation, the cells under the cholesterol-agar medium were dead, but the cells under lecithin-agar medium just began to slough off. The cells treated with collagen and β-carotene began to deteriorate at this time. Because these changes in the cells appeared at 80 to 96 hr, the plaques were read within 48 to 72 hr in all cases to minimize the involvement of the undesirable effects.

### DISCUSSION

Thermal inactivation of poliovirus strains has been studied by a number of investigators. Reports by Youngner (18) and Papevangelou and Youngner (15) indicated that the strain variants of poliovirus can have different thermal inactivation kinetics. Dulbecco (5) has designated these strains of viruses as temperature-sensitive or temperature-resistant. The type strain investigated in this study was, upon examination, most sensitive to an inactivation temperature of 50°C.

Previous thermal inactivation experiments with a heat-sensitive strain of poliovirus in the presence of 1-cystine (16) resulted in stabilization of the virus when it was preincubated with the amino acid for various periods of time at 37°C. This report suggested that added compounds could stabilize the virus during heat treatment. In the experiments reported here, collagen also appeared to exert a protective effect (Table 2). A feasible explanation of this stabilization could be that the collagen absorbs the thermal energy and prevents the denaturation of the viral protein coat. This may happen by entrapment of the virus in the collagen matrix, the integrity of the virion being conserved.

Besides the possible loss of infectivity due to thermal denaturation of the protein coat of the virion, loss of the infectivity due to destruction of the attachment sites with subsequent loss of infectivity, as suggested by Graham (9), could also be an explanation. Examination of the thermally inactivated virus by ultraviolet from 220 to 360 nm revealed a minimal change in εₘₐₓ at 280 nm. This was suggestive of some mechanism of inactivation other than denaturation of the virus protein. In fact, there was residual infectivity in the extracted naked viral RNA and the denaturation kinetics of poliovirus RNA have been reported to follow first-order kinetics (7, 8, 14). Whichever of the mechanisms was operative, it is evident that infectious material was still present in the inactivated particles.

It is suggested from the results presented here that the presence of different food constituents may influence the stability of a potential infectious particle against thermal inactivation during heat treatment of the infected food. The observation (Table 3) that cholesterol and lecithin can render cells more susceptible to infection with the virus suggests that the immediate past history of the cell that will be a candidate for virus invasion could be important.

**Table 3. Preinoculation effect of selected organic compounds on poliovirus infectivity in Hela cells**

| Expt | Compound | Residual PFU/culture |
|------|----------|----------------------|
| 1    | Collagen | 164 ± 9              |
|      | Lecithin | 264 ± 12             |
|      | β-Carotene | 109 ± 4              |
|      | Cholesterol | 318 ± 11             |
|      | Control  | 128 ± 6              |
| 2    | Collagen | 157 ± 10             |
|      | Lecithin | 263 ± 13             |
|      | β-Carotene | 108 ± 2              |
|      | Cholesterol | 316 ± 8              |
|      | Control  | 134 ± 4              |

* Virus suspension was diluted so that the control and test virus would give approximately 100 plaques per 60-cm² culture. No heat was applied to the virus suspension in these experiments.
In addition, the pretreatment of cells with cholesterol or lecithin before inoculation had a decided effect on the longevity of the cells. The controls treated in a similar manner without inoculation with the virus remained healthy in appearance and viable longer than the untreated controls. This increase in infectivity and the observed cellular longevity were similar to observations with cytokinin-treated and untreated poliovirus-infected, uninfected HeLa P cells (Virology, in press).

Because of the relatively long incubation period of some of the infectious particles in a host before expression of the virus occurs, it would be difficult to trace the virus back to its source; also, other possible virus contaminants that could be introduced into canned foods, such as enteroviruses or infectious hepatitis viruses (6), would require more extensive thermal treatment to inactivate the total virus population. In addition, foods containing collagen or cholesterol possibly contaminated with viruses, specifically enteroviruses, should be thermally treated so as to destroy the infectious particles. Therefore, it is suggested by these experiments that foods rich in each of the above-tested organic compounds could alter the interaction between the virus and the cell or stabilize the virus aggregates, thereby preventing complete destruction of the virus during thermal treatment of processed (heat) foods.

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