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Potential Sources of Error During Virus Thermal Inactivation

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A review of virus thermal inactivation data published in the literature demonstrated variations in reported virus resistance. Examination of the methods used indicated that numerous studies were made by heat processing virus suspensions in test tubes. Duplication of some of the methods using milk suspensions of poliovirus 1 showed virus persistence after heating as a result of uneven temperature distribution inside the test tubes. Unless the containers (preferably sealed ampoules or capillary tubes) are completely submerged in the water bath and agitated vigorously, apparent virus persistence may be encountered.

A survey of 135 articles on virus thermal inactivation indicated that there was considerable variation in the reported thermal resistance of viruses (3). Examination of the materials and methods used in these studies showed that these variations were probably related to: (i) the concentration of viruses, (ii) the menstrua in which the viruses were processed, and (iii) the methods used in the study. In 58 of the references pertaining to inactivation in fluid media, 30 investigators reported using a water bath to inactivate viruses, and 21 of these investigators used test tubes to process the viral suspensions. There were no standard procedures for the test tube methods. A study was designed to duplicate the test tube procedures used by a number of investigators and to determine whether such procedures would affect the data obtained in virus thermal inactivation studies.

MATERIALS AND METHODS

Virus. Poliovirus 1 (LSc2ab) was propagated in Vero cells, dispensed into vials, and stored at −60°C until used.

Tissue culture. Buffalo green monkey (BGM) cell cultures obtained from the American Type Culture Collection, Rockville, Md., were used in all tests.

Virus assay. A plaque-forming unit assay system (4), which consisted of an agar medium overlay and monolayers of BGM cell sheets (45 cm^2) in 6-ounce (approximately 0.17-liter) bottles, was used to monitor virus numbers.

Samples. Samples were suspended in sterile whole milk and raw milk.

Equipment. A 10-liter, heavy-wall glass container equipped with a Bromwell constant-temperature heater (temperature variance of ± 0.01°C) was used as the water bath. A shaking water bath (125 strokes per min) was substituted when necessary.

Modified Scharer phosphatase test. The modified test is similar to the standard method (2), except that the color is extracted from the sample with cold *n*-butyl alcohol and compared to standards by use of a colorimeter or spectrophotometer (unpublished data, G. P. Murthy, Division of Microbiology, Bureau of Foods, Food and Drug Administration).

Thermal processing procedures. Temperatures were monitored by introducing a thermocouple wire (30-gauge copper constantan) through holes punctured in the sides of glass, screw-cap test tubes (20 by 125 mm). A thermocouple was placed inside separate tubes at different points so as to measure the temperature of the glass, air, and liquid inside the tubes. The space around the wires was sealed with epoxy cement. All holes were made at points below water bath level to eliminate possible air-cooling of the thermocouple wires, which could result in lower-than-actual temperature readings. Entering the thermocouples through the sides of the tubes allowed for placement of magnetic stirring bars in the bottoms of the test tubes. The temperature at the various positions was monitored by a 12-point Speedomax recorder.

The screw-cap test tubes, containing 10 ml of milk, were placed in a rack and secured by elastic bands. Paper liners were replaced by rubber liners in all tubes to prevent leakage. Magnetic stirring bars were inserted in some tubes to stir the fluid contents. The rack of tubes was placed in a 72°C constant-temperature water bath. Either the tubes were completely submerged in the bath, or the water level of the bath was adjusted to 0.5 inch (1.3 cm) above the level of the milk in the test tubes. In some trials, the tubes were submerged in a horizontal position by turning the rack sideways in a shaking water bath. This position allowed the milk to flow back and forth so as to continuously wash the insides of the tubes.

In the virus inactivation and phosphatase studies, the tubes were placed in an ice bath immediately after heating and agitated in the bath for rapid cooling.

RESULTS

The first phase of the study was designed to determine whether the tube position in the water bath and the mixing of the milk suspension

would affect the time reported by investigators in the virus inactivation articles. When the test tubes were placed in the normally used upright position, the time required for the milk to reach bath temperature (72°C) varied from 132 to 156 s (Fig. 1). Plotting the data on inverted semilog paper, a common practice used by engineers, resulted in a straight line. When the rack was

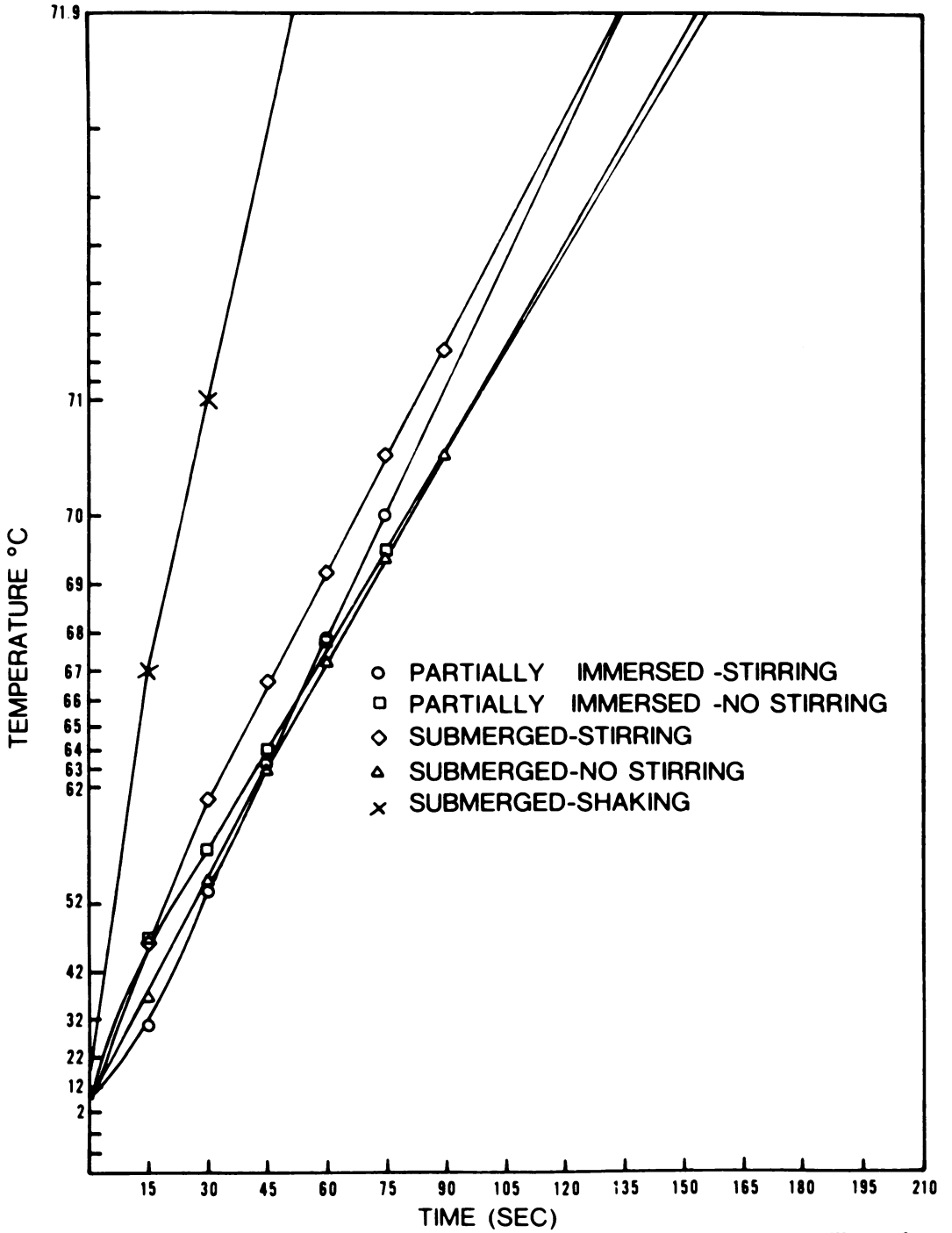

FIG. 1. *Effect of tube position in the water bath and agitation on the time required for the milk sample to reach bath temperature.*

turned on its side and placed in a shaking water bath, the time required for the submerged tubes to reach bath temperature was reduced to 51 s. None of the investigators had reported using this technique.

In the second phase of the study, the temperatures of the milk, air, and glass surfaces inside the test tube were determined when the water bath level was 0.5 inch (1.3 cm) above the level of the milk in the tube (Fig. 2). This procedure was used by the majority of the investigators. Only the milk and the glass below the water level of the bath reached bath temperature.

A number of methods have been reported for adding the viruses to the fluid suspensions or mixing the suspensions to attain even distribution of the viruses. If any of these procedures resulted in deposition of even a small portion of the suspension on the glass surfaces above the fluid level, survival of the viruses could have occurred. Figure 3 shows the results of mixing virus suspensions by inverting the tube before heating. The fluids never completely drained back into the milk, and viruses were left on the glass surfaces above the level of the milk in the tubes.

The heating times shown in Fig. 1 (51, 132, and 156 s) indicate the time required to attain bath temperature by each of the three methods. Samples were removed at 15-s intervals and immediately cooled in an ice bath. Viruses in the inverted, partially immersed tubes were not inactivated even after 180 s. Although viruses in the completely submerged tubes were inactivated, those in contact with the inner cap liners probably did not reach bath temperatures in the submerged-static procedure because of lower heat transfer through the cap and liner.

Problems associated with uneven heat distribution or contamination of the inside surfaces or cap liners of the test tubes were demonstrated by using the phosphatase test to monitor the samples. This procedure accurately determines whether all portions of the milk have reached the thermal exposure times required to reduce the phosphatase enzyme to pasteurization levels. A study was made duplicating many of the test tube positions and mixing procedures previously

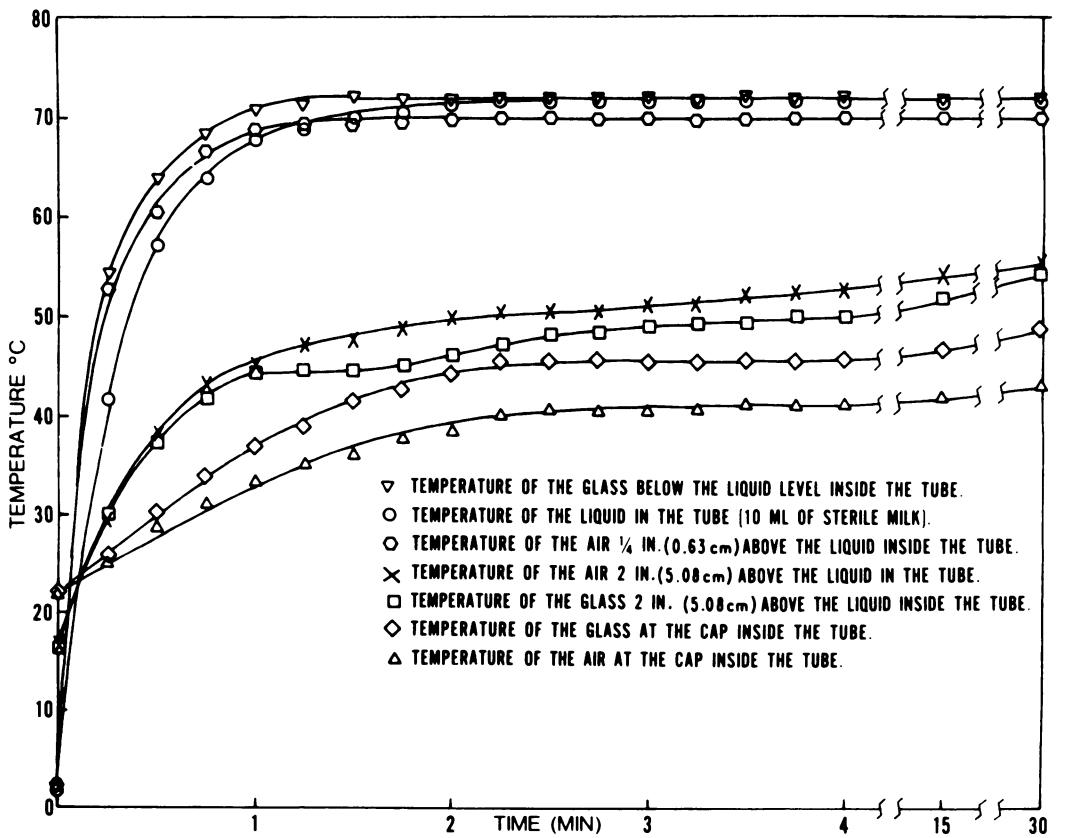

FIG. 2. *Temperatures attained by the milk, air, and glass surfaces of test tubes heated in a water bath at 72°C.*

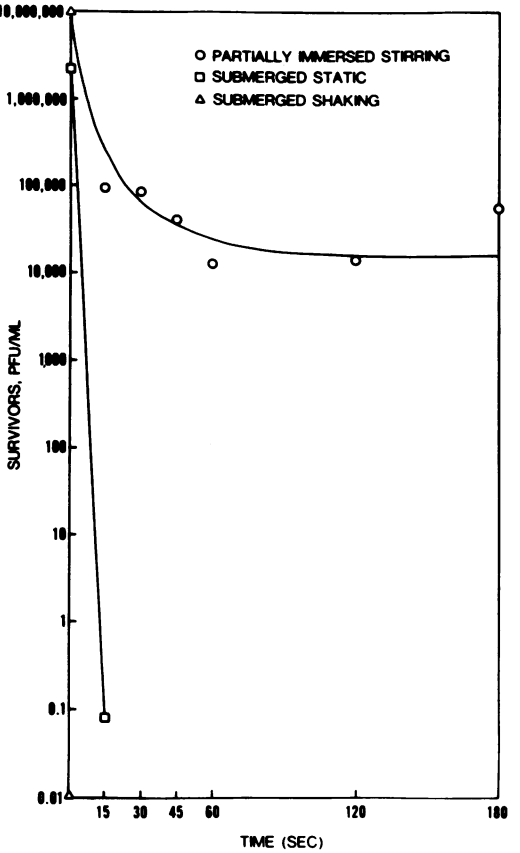

FIG. 3. *Variation in thermal inactivation of poliovirus 1 resulting from tube position and agitation of test tubes inverted once before heating at 72°C. The tubes were heated for the 51, 132, and 156 s (Fig. 1) required to reach bath temperature before timing the process.*

described (Table 1). The only samples showing acceptable pasteurization levels were the tubes that were submerged in a horizontal position and mixed in the shaking water bath. The use of screw-cap test tubes is not recommended for thermal inactivation studies because of the possibility of leakage or contamination in the cap area. This problem can be eliminated by the use of sealed ampoules or capillary tubes.

DISCUSSION

The methods used by investigators may result in variations in reported thermal resistance data. In instances when low temperatures are used to determine rates in virus reduction or in studies attempting to explain virus thermal kinetics, low-level virus survival may be of little importance. But in thermal processes designed to inactivate pathogens and protect the consumer, virus survival is significant.

This study has shown that the time required to inactivate a virus suspension depends on the procedures used to heat the milk in the test tube. In processes requiring a long exposure at a specific temperature, a difference of a few minutes below temperature would probably be of little significance. However, where high-temperature, short-time methods are used, a difference of a few seconds may result in success or failure of the process.

An example is shown in Fig. 1. If the total heating time was 210 s, the submerged-shaking test tubes processed in the shaking water bath reached bath temperature (72°C) in 51 s and were at temperature for the remaining 159 s. Partially immersed tubes, not stirred by the magnetic stirring unit, required 156 s to reach

TABLE 1. *Factors affecting pasteurization of milk samples in test tubes processed at 72°C for 15 s*

Expt no.	Tube position[a]	Special conditions	Phenol (μg)	Pasteurized
1	P1	Drop of raw milk placed at tube rim after heating	>20.0	−
2	P1	Drop of raw milk placed at tube rim before heating	4.66	−
3	P1	Tube inverted to mix sample before heating	11.13	−
4	P1	Agitated with magnetic stirrer during heating	9.43	−
5	S	Not agitated during heating	3.3	
6	S	Agitated with magnetic stirrer during heating	1.5	−
7	S	Shaken in horizontal position, 125 strokes per min	0.33[b]	+
8	Control	No treatment	>20.0	−

[a] P1, Partially immersed; liquid level in the bath was 0.5 inch (1.3 cm) above the level of the milk in the tubes. S, Submerged; the tubes were anchored to the racks, and the tops of the tubes were at least 2 inches (5.2 cm) below the level of the liquid in the bath.

[b] <1.0 μg of phenol was considered pasteurized when the improved Scharer phosphatase test was used.

bath temperature, and the tubes were only at temperature (72°C) for 54 s.

The lethality of the processes may be calculated. If a z value of 4°C is used and the data are transported at 15-s intervals (Fig. 1), the lethal rates may be obtained from Fig. 4. The 4°C z value was considered a fair estimate for a typical virus suspension after analysis of laboratory-acquired z values for four virus suspensions (5). A z value of 4°C means that 4° of the temperature scale was used to pass through 1 log cycle. The average temperature was obtained for each of the 15-s intervals. For example, an average temperature of 69.5°C was estimated for the second 15-s interval of curve X. A temperature of 69.5°C has a lethal rate of 0.240 (Fig. 4). The interval was 15 s. Therefore, 0.240 × 15 s = 3.60 s for the lethality for the second 15-s interval. This means that the lethality produced during the 15-s interval was equivalent to that which would be produced at 72°C for 3.6 s. The lethality for each of the 15-s intervals was calculated and added to obtain the cumulative F. The lethality of the submerged shaking process was ~177 s at 72°C, whereas that of the partially immersed, nonagitated process was ~115 s. If the total heating times were as projected in Fig. 1 (210 s), the submerged tubes were subjected to the lethal effect of the 72°C bath temperature for 84% of the time, and the other set of tubes for only 55% of the time. The lethality resulting from cooling was not calculated because the rapid reduction in temperature when the tubes were plunged into the ice bath made such calculations insignificant.

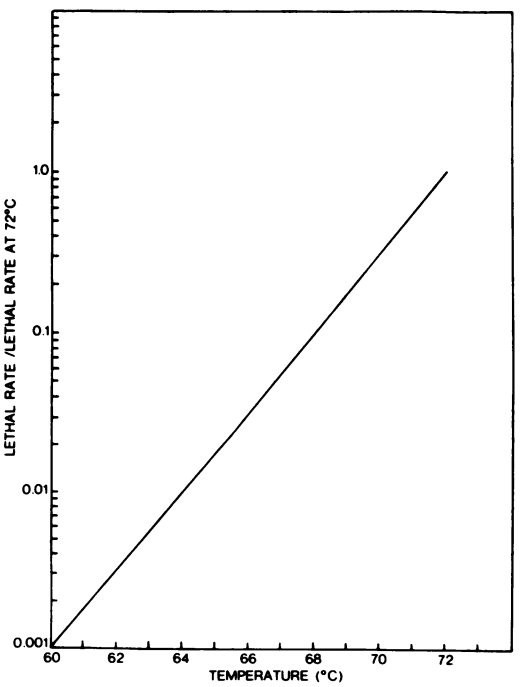

FIG. 4. *Lethal rate versus time plot with a z value of 4°C used to calculate the lethality of each of the 15-s intervals shown in Fig. 1.*

Mixing of the virus suspension during heat processing is essential to ensure that all virus particles are subjected to the processing temperature. In Fig. 2 the temperatures shown for the air and glass surfaces demonstrate the importance of effective mixing and of heating all portions of the tube by complete submersion. In early studies on Q fever, even a slight difference in temperature at the surface of the milk in vat pasteurizing units was sufficient to prevent destruction of rickettsiae in the foam (1). This problem was overcome by the introduction of an airspace heater that maintained the temperature of the air at a level slightly higher than that of the milk.

A number of procedures such as inversion of the tube, pipetting to mix the sample, splattering during mixing, touching the side of the tube with the pipette, or pushing solid or semisolid inocula to the bottom of the test tube may result in virus retention on the sides of the tube. These viruses could be heated to a lower temperature than the viruses in the fluid suspension and thus be a source for recontamination of the sample. An example is shown in Fig. 3, where inversion of the tube to mix the sample, a frequent practice of chemists and microbiologists, resulted in contamination of the cap and sides above the liquid in the tube and apparent virus resistance to the thermal process. The 51, 132, and 156 s (Fig. 1) required to reach bath temperature were used before timing the samples.

Research on the thermal resistance of viruses would be of greater benefit to scientists, industry, and regulatory agencies if the thermal process could be designed to ensure that every particle of the suspension and all inner surfaces of the container were subjected to the time and temperature under study. Information thus obtained could be used to determine the safety of thermal processes and to compare variations in virus thermal resistance.

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