APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1983, p. 446–451
0099-2240/83/080446-06$02.00/0

Vol. 46, No. 2

Effect of Heat on Virus Inactivation by Ammonia

W. D. BURGE,[1]* W. N. CRAMER,[2] AND K. KAWATA[3]

U.S. Department of Agriculture, Agricultural Research Service, Biological Waste Management and Organic Resources Laboratory, Beltsville, Maryland 20705[1]; Maryland Environmental Service, Annapolis, Maryland 21401[2]; and Department of Environmental Health Sciences, The Johns Hopkins University, Baltimore, Maryland 21205[3]

Received 28 December 1982/Accepted 6 May 1983

The rate of inactivation of bacteriophage f2 and poliovirus 1 (CHAT) by NH_3 was strongly influenced by temperature. The process was pseudo-first order at all temperatures and NH_3 concentrations. Poliovirus was inactivated at a greater rate than f2, but the change in the rate of inactivation with increasing temperature in the range of approximately 10 to 40°C was greater for f2 than for poliovirus. At higher temperatures, the rate of change was greater for poliovirus. Arrhenius plots of the data were biphasic, indicating that two inactivation processes were occurring, one for the low temperature range and another for the high temperature range. However, the magnitudes of the thermodynamic variables for f2 were low enough, as calculated for the low (10 to 35°C) and high (35 to 60°C) phases, that inactivation could have occurred by breakage of nucleic acid chains. For poliovirus, the sizes indicated possible involvement of nucleic acid at the low temperatures (10 to 40°C) but some unknown mechanism for the high temperatures (40 and 50°C).

Ammonia has been shown to be virucidal in sludge and in buffer solutions (2, 5, 9, 10). In addition, the kinetics of poliovirus 1 (CHAT) and f2 inactivation by NH_3 have been reported to be pseudo-first order at 20°C (2). In studies to determine the mechanism of inactivation, NH_3 was found to cleave the RNA of intact poliovirus 1 (CHAT) particles but not the RNA isolated from the poliovirus particles (9). Of seven viruses (three strains of poliovirus, two strains of coxsackievirus, and one strain each of echovirus 11 and reovirus 3), only one, the reovirus, was relatively resistant to inactivation by NH_3 (10). All have single-stranded RNA, except for the reovirus, which has double-stranded RNA. Heating has been shown to increase the rate of viral inactivation by NH_3 in sludge, but the relationship between heat and NH_3 inactivation has not been described mathematically (5, 10).

When applied to the thermoinactivation of viruses, the magnitude of the energy of activation (E), the changes in the enthalpy of activation (ΔH_+^+) and the entropy of activation (ΔS_+^+) can be used to help deduce the nature of the reaction (6, 11). For single-stranded RNA viruses, large values indicate that the viral coat is the site of inactivation through protein denaturation; smaller values may mean that RNA is the site through rupture of the RNA chain. For inactivation by NH_3, small values would tend to reinforce Ward's (9) observation that RNA cleavage is involved in poliovirus inactivation, whereas large values would tend to indicate that RNA cleavage is incidental.

In this study, the kinetics of f2 and poliovirus 1 (CHAT) inactivation were examined at 10 and 30°C as influenced by NH_3 concentration. To extend further the study of the effect of temperature, the kinetics of inactivation for f2 were followed at 40, 50, and 60°C, and the kinetics for the poliovirus were followed at 40 and 50°C at one NH_3 concentration (300 ppm [300 mg/liter]). The data obtained for the effect of temperature, together with those at 20°C from a previous study (2), were used to determine E, ΔH_+^+, and ΔS_+^+, ΔG_+^+. The results show that both viruses have a lower energy of activation for the lower temperature range (10 to 35°C for f2 and 10 to 40°C for poliovirus) than they do for the higher temperature range tested. For both viruses, the thermodynamic state variables calculated from the energy of activation indicate that the NH_3 inactivation process results from a rupture of the RNA chains in the lower temperature range. For the higher temperature range, the calculated values indicate a change from RNA as the site for poliovirus. For f2, despite an increase in activation energy, the magnitude of the thermodynamic state variables was still low enough to implicate RNA in the inactivation process.

MATERIALS AND METHODS

Preparation of virus and cells. The CHAT strain of poliovirus 1 (ATCC VR-192), f2 bacterial virus (ATCC

15766-B), and its host *Escherichia coli* K-13 (ATCC 5766) were obtained from the American Type Culture Collection, Rockville, Md. Poliovirus 1 was grown in serially propagated Buffalo green monkey kidney cells obtained from Microbiological Associates, Walkersville, Md., in the 24th passage. The procedures for growing, concentrating, purifying, and plaquing both viruses have been reported previously (2).

NH_3 solutions. The Henderson-Hasselbalch equation:

$$pH = pK_1 + \log \frac{(NH_3)}{(NH_4^+)} \quad (1)$$

was used to calculate the amount of NH_4Cl needed to be added to sterile 0.1 M Tris made with NH_3-free water to obtain the desired NH_3 concentrations at various pH levels. The concentration actually achieved was determined with an Orion NH_3 electrode, using standard curves as previously reported (2).

Experimental procedure. In all studies, 199 ml of the NH_3 working solution were poured into a sterile 250-ml Erlenmeyer screw-capped flask. One milliliter of an appropriate dilution in distilled water of the purified f2 or poliovirus stock solutions was added to give a titer in the flask of from 1.0×10^6 to 2.0×10^7 PFU/ml. The flasks had been allowed to equilibrate at the experimental temperature before the virus was added. The control flask was prepared in the same way, except that the NH_4Cl was replaced by an equivalent amount of NaCl so that the ionic strengths of both would be identical. The contents of the treatment and control flasks were mixed quickly after virus addition and sampled with time during incubation to determine the virus titer, pH, and ammonia level at the experimental temperature.

The fraction of viruses surviving as corrected for losses in the control was calculated by dividing the number of virus particles surviving exposure to NH_3 by the number surviving in the control flask during the same time interval. All experiments were repeated, and the average values from the two studies were used.

In these studies, the NaCl and NH_4Cl solutions were adjusted to pH 8.0. The bacterial virus was exposed to NH_3 levels of 25, 50, 150, and 400 mg/liter at 10°C and 40, 100, 200, and 600 mg/liter at 30°C. Poliovirus was dosed with 50, 100, 300, and 800 mg of NH_3 per liter at both temperatures. For the higher temperature studies of 40, 50, and 60°C for f2 and 40 and 50°C for poliovirus, an NH_3 concentration of 300 mg/liter was used.

For thermodynamic calculations, the inactivation rate constants (k) for 300 mg of NH_3 per liter were used. Since this concentration was not used in the kinetic studies for f2 at 10 and 30°C, the k for 300 mg/liter was calculated from the relationship experimentally obtained for k and NH_3 concentrations actually used.

RESULTS

Survival curves. The dependence of the rate of inactivation on NH_3 concentration of f2 (Fig. 1) and poliovirus (Fig. 2) is evident at the temperatures of 10 and 30°C. The curves are highly linear, indicating the pseudo-first-order nature of the reaction as discussed in a previous paper (2), for the effect of NH_3 at 20°C on these two viruses. The inactivation rates for f2 at 40, 50, and 60°C and for poliovirus at 40 and 50°C were also clearly pseudo-first order, as shown by the

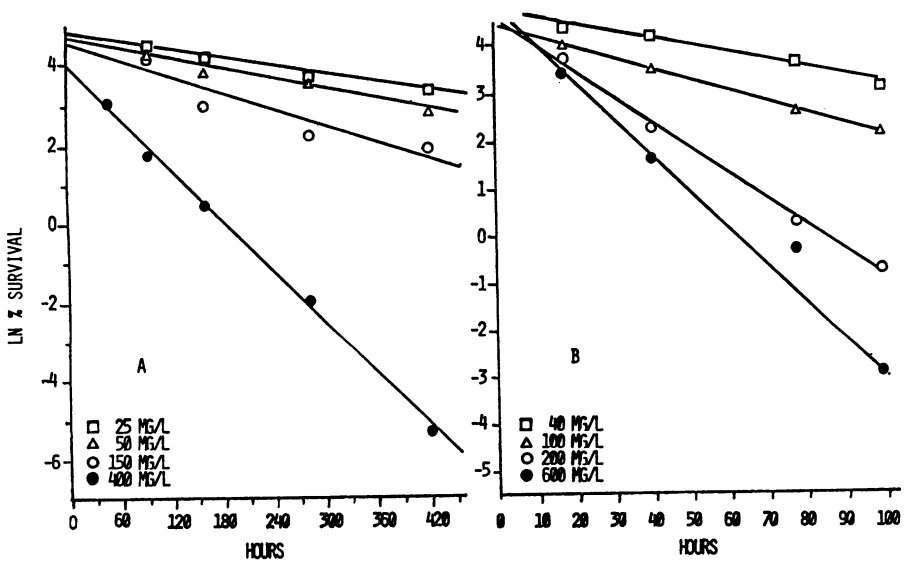

FIG. 1. Inactivation of f2 at 10°C (A) and 30°C (B) in NH_4Cl solutions containing NH_3 concentrations as shown.

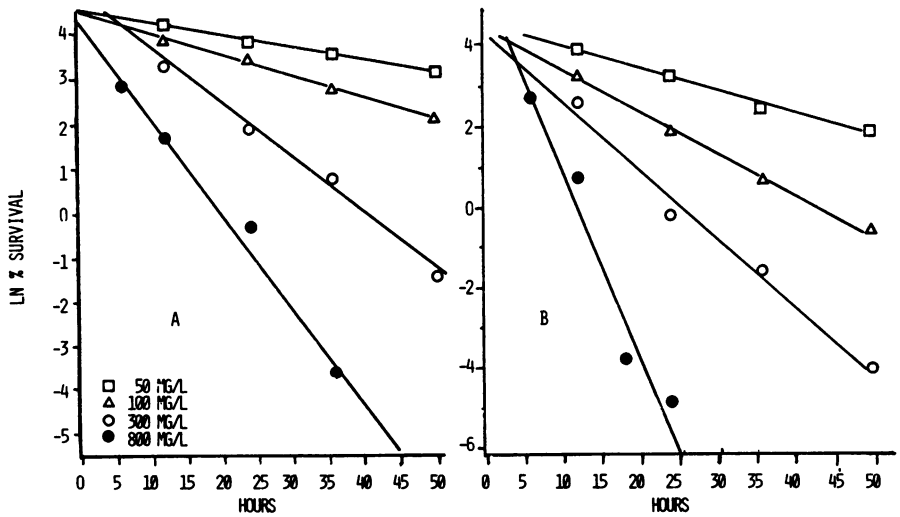

FIG. 2. Inactivation of poliovirus 1 (CHAT) at 10°C (A) and 30°C (B) in NH_4Cl solutions containing NH_3 concentrations as shown.

linearity of the curves resulting from semilog plots (Fig. 3 and 4, respectively).

Effect of NH_3 concentration and temperature on *k*. For both f2 and poliovirus, a plot including the 20°C data (2) of log k as a function of log (NH_3) produced a family of approximately parallel lines (Fig. 5). F tests for the linearity of the log regressions showed that they were all highly significant. No interaction between NH_3 concentration and temperature was found when the slopes of the six lines were compared, indicating they could not be considered other than parallel ($P = 0.02$).

It is apparent from Fig. 5, as from the survival curves, that f2 is more resistant to NH_3 than is poliovirus. Even at 30°C, the k values for f2 were less than those of poliovirus at all three temperatures studied. This can be shown another way by comparing the times needed by the two viruses for 99% inactivation at common NH_3 concentrations and temperatures. For this purpose, the k values are divided into 4.606 (4). As an example of the difference in the sensitivity of the viruses at 20°C and 100 mg of NH_3 per liter, 363 h were needed for inactivation of f2, compared with only 71 h for poliovirus 1.

Although f2 was more resistant to inactivation than was poliovirus 1, the change in the rate of inactivation was greater with increasing temperature for f2 than for poliovirus in the lower temperature range. This is evident from the wider spacing in the lines relating to f2 than for poliovirus in Fig. 5 but can be more clearly shown by calculation of the Q_{10} values by the following equation:

$$Q_{10} = \frac{k_2}{k_1}^{\exp \frac{10}{T_2 - T_1}} \quad (2)$$

in which k_1 and k_2 are inactivation rate constants for T_1 and T_2, respectively, as expressed in degrees absolute. The resulting Q_{10} values for f2

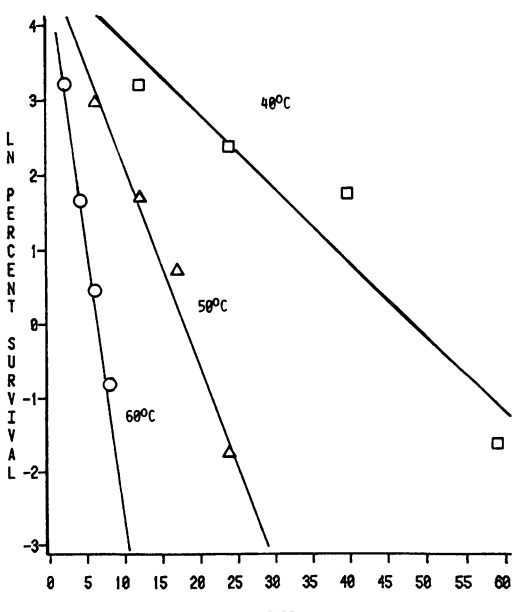

FIG. 3. Inactivation of f2 at 40, 50, and 60°C at an NH_3 concentration of 300 mg/liter.

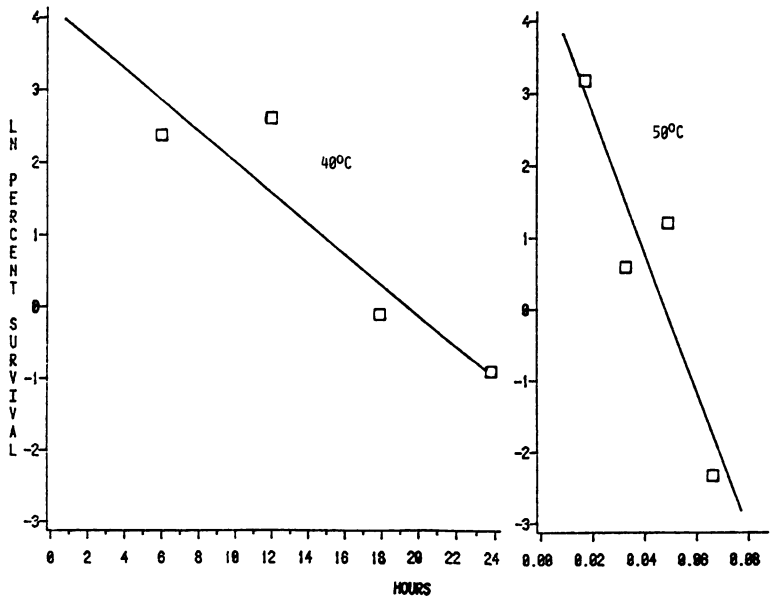

FIG. 4. Inactivation of poliovirus 1 (CHAT) at 40 and 50°C at an NH_3 concentration of 300 mg/liter.

at 300 mg of NH_3 per liter ranged from 1.57 between 10 and 20°C to 2.08 between 20 and 30°C, with an average of 1.82. The values for poliovirus 1 under the same conditions were 1.13 and 1.20, respectively, with a mean of 1.16.

Thermodynamics. The relationship between the rate of reaction constant and temperature can be seen in the Arrhenius equation shown in its integrated form:

$$\log k = -E/RT + C \quad (3)$$

in which T is the absolute temperature, E is the energy of activation, and R is the molar gas constant (1.99 cal/°C [8.33 J/°C]). When log k is plotted against $1/T$, a straight line is generated with a slope of $-E/R$ and an intercept C on the log k axis.

Further, in accord with Eyring's theory of absolute reaction rates (3), the change in the enthalpy of activation (ΔH_+^+) is a function of E as follows:

$$\Delta H_+^+ = E - RT, \quad (4)$$

and ΔS_+^+ is a function of ΔH_+^+

$$\Delta S_+^+ = R \log \frac{k N_0 h}{ZRT} + \frac{\Delta H_+^+}{T} \quad (5)$$

$$\Delta G_+^+ = \Delta H_+^+ - T\,\Delta S_+^+ \quad (6)$$

wherein N_0 is Avogadro's number, h is Planck's constant (6.625×10^{-27} erg-s), and Z is the transmission coefficient, usually taken to be 1.

Arrhenius plots for f2 and poliovirus (Fig. 6) as made for an NH_3 concentration of 300 mg/liter show that inactivation by NH_3 is biphasic. In the lower temperature range, the E value is

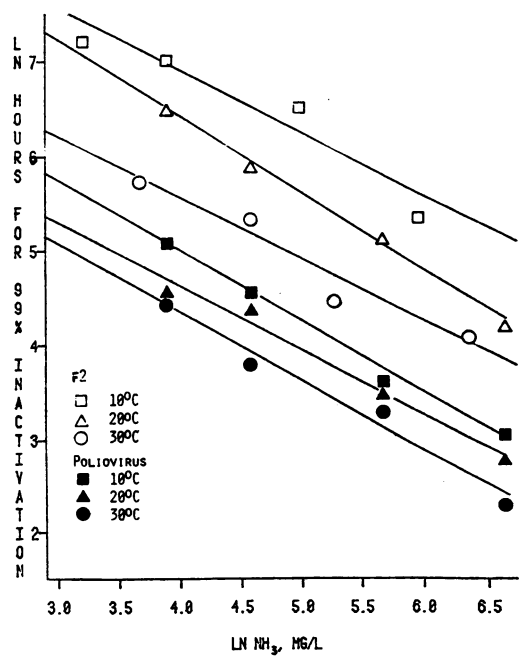

FIG. 5. The relationship between the inactivation rate constant (k) and NH_3 concentration as influenced by temperature.

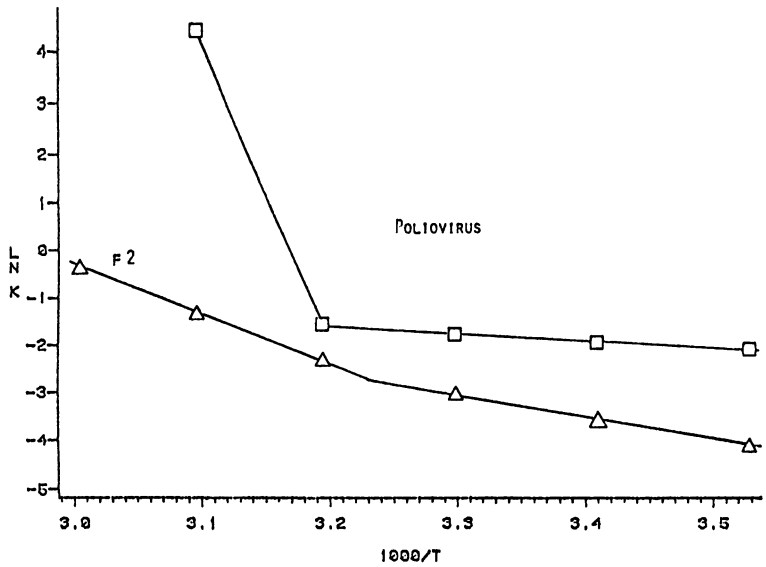

FIG. 6. The relationship between the inactivation rate constant (k) and temperature (degrees Kelvin) for f2 and poliovirus 1 (CHAT).

higher for f2 as indicated by a greater slope than for poliovirus. At the higher temperature phase, however, the situation changes dramatically, with a much greater slope (higher E) for poliovirus. The values for E and the thermodynamic state variables ($\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$, and $\Delta G^{\ddagger}$) as calculated by the use of equations 3, 4, and 5, 6, from the slopes of the curves of Fig. 6 are given in Table 1.

DISCUSSION

Values from the determination of the thermodynamic state variables can be used to help deduce the nature of a reaction. In the case of virus inactivation by heat, Woese (11) states that for many of the simple virus particles, the process reduces to either damage to receptor sites through denaturation of the protein coat or damage to nucleic acid; or damage to both the coat and nucleic acid may be involved. The inactivation of protein and DNA is associated with high $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values because it is associated with the rupture of a number of hydrogen bonds whose breakage results in a collapse or drastic refolding of the secondary structure of the molecules. Such a complex process might be expected to be higher than first order. However, heat inactivation studies (7) have shown that it is first order for the heat denaturation of a number of enzymes, although not for all.

The energy involved is lower for single-stranded RNA because the rupture of one diphosphoester bond linking nucleotides will cleave the molecule. For DNA and double-stranded RNA, the rupture of at least two bonds appears to be needed within a span of not more than two nucleotides (10). The $\Delta H^{\ddagger}$ accompanying inactivation of single-stranded RNA may be on the order of 10-fold lower than for DNA and protein, and the $\Delta S^{\ddagger}$ values may not only be much lower but may be negative.

Negative $\Delta S^{\ddagger}$ values indicate a loss in degrees of freedom, i.e., formation of a less probable structure due to molecular rearrangements or new bonds formed during the rupture of the old ones. Brown and Todd (1) discussed a possible explanation of negative $\Delta S^{\ddagger}$ values accompanying the inactivation of single-stranded RNA. Hydrolytic cleavage of the diphosphoester nucleotide linkage is preceded by formation of a triphosphoester involving a reaction with the C_2'–OH, a group not present in DNA molecules.

The reasoning described above is not totally applicable to inactivation by NH_3, because an inactivation reaction not involving RNA breakage (such a reaction might be one to block a receptor site in the virus protein coat) might proceed with relatively low values for the thermodynamic variables. However, the low values for $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ make it not possible to rule out rupture of RNA as a possible mechanism at all temperatures for f2 and for poliovirus below 40°C. Therefore, these results lend support to, or at least do not contradict, Ward's (9) proposal that RNA rupture is the mechanism for inactivation. For poliovirus at temperatures above 40°C, the large $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values make it seem likely that inactivation occurs by some other process.

TABLE 1. Rate constants for temperatures used and corresponding thermodynamic state variables characterizing the biphasic inactivation of bacteriophage f2 and poliovirus 1 (CHAT) by NH_3 at a concentration of 300 mg/liter

Virus	Temp (°C)	k (h^{-1})	E (kJ/mol)	$\Delta H^{\ddagger}$ (kJ/mol)	$\Delta S^{\ddagger}$ (J/deg per mol)	$\Delta G^{\ddagger}$ (kJ/mol)
f2	10	0.0160	38.94	15.32	−293.55	98.43
	20	0.0274		14.48	−294.13	100.70
	30	0.0488		13.69	−294.01	102.83
	40	0.0995	87.25	61.13	−138.42	104.46
	50	0.2699		60.29	−139.05	105.97
	60	0.7335		59.45	−138.92	105.72
Poliovirus	10	0.1245	13.35	11.01	−292.21	93.74
	20	0.1434		10.93	−292.75	96.76
	30	0.1719		10.84	−292.79	99.61
	40	0.2145	259.00	256.41	491.97	102.33
	50	96.5623		256.33	556.45	76.49

The values obtained for poliovirus for the high temperature range might be criticized on the basis that the slope utilized for calculation of E was obtained from a line drawn through only two points, with the lower point appearing to fit well into the low temperature phase data (Fig. 6). However, if another point were obtained between the 50 and 40°C points, it could only have increased the slope. (An attempt was made to obtain survival data at 60°C for poliovirus, but the inactivation rate was too rapid to obtain satisfactory measurements.) An increase in the slope would increase the values for the change in enthalpy and entropy of activation, further supporting the conclusion that RNA rupture was not involved in the inactivation process.

The hypothesis that the relatively low energies observed in the inactivation of f2 throughout the entire temperature range studied—and in poliovirus in the lower range studied—are associated with the vulnerability of single-stranded RNA is consistent with the observation by Ward and Ashley (10). They observed, in an NH_3 inactivation study using four single-stranded RNA viruses and one double-stranded RNA virus, that only the double-stranded RNA virus, a reovirus, was resistant to inactivation. This implies that DNA viruses, because of their double-stranded nucleic acid, may also be resistant to inactivation by NH_3. Further, the kinetics for double-stranded RNA and DNA viruses should be other than first order because at least two ruptures, one in each of the two nucleotide chains, are needed (8). The NH_3 as produced in sewage sludge by ammonification, together with the elevation of pH by the addition of lime and moderate elevation of temperature, has promise as a very effective and economical way of inactivating viruses. However, the possibly higher resistance of double-stranded nucleic acid viruses to NH_3 inactivation must be taken into consideration.

ACKNOWLEDGMENTS

This research was partially supported by funds from the Maryland Environmental Service, Annapolis, Md.; the District of Columbia Department of Environmental Services; and the U.S. Environmental Protection Agency, Office of Research and Development.

Review of this manuscript and assistance in statistical analyses by N. K. Enkiri are gratefully appreciated.

LITERATURE CITED

1. **Brown, D. M., and A. R. Todd.** 1955. The nucleic acids. Academic Press, Inc., New York.
2. **Cramer, W. N., W. D. Burge, and K. Kawata.** 1983. Kinetics of virus inactivation by ammonia. Appl. Environ. Microbiol. **45**:760–765.
3. **Daniels, F., and R. A. Alberty.** 1963. Physical chemistry. John Wiley & Sons, Inc., New York.
4. **Fair, G. M., and J. C. Geyer.** 1954. Water supply and wastewater disposal. John Wiley & Sons, Inc., New York.
5. **Fenters, J., J. Reed, C. Lue-Hing, and J. Bertucci.** 1979. Inactivation of viruses by digested sludge components. J. Water Pollut. Control Fed. **51**:689–694.
6. **Ginoza, W.** 1958. Kinetics of heat inactivation of ribonucleic acid of TMV. Nature (London) **181**:958–961.
7. **Greenberg, D. M., and T. Winnick.** 1940. Plant proteases. III. Kinetic properties. J. Biol. Chem. **135**:781–787.
8. **Thomas, C. A., Jr.** 1956. The enzymatic degradation of deoxyribose nucleic acid. J. Am. Chem. Soc. **78**:1861–1868.
9. **Ward, R. L.** 1978. Mechanism of poliovirus inactivation by ammonia. J. Virol. **26**:299–305.
10. **Ward, R. L., and C. S. Ashley.** 1977. Identification of the virucidal agent in wastewater sludge. Appl. Environ. Microbiol. **33**:860–864.
11. **Woese, C.** 1960. Thermal inactivation of animal viruses. Ann. N.Y. Acad. Sci. **81**:741–750.