ganisms and without cross-resistance to the majority of other clinically useful antibiotics. Oleandomycin is an agent which, with the exception of cross-resistance to members of the erythromycin group, displays these properties.

Summary (1) *In vitro* under the specific conditions of the gradient plate technic, combinations of sulfisoxazole and oleandomycin in specific ratios show an activity superior to that observed with the single constituents. (2) Supplemental activity was also observed *in vivo* in the streptococcal, staphylococcal and pneumococcal infections when the ratio of the combination of sulfisoxazole to oleandomycin was 5 to 1. (3) The same type of effect was also observed in *Salmonella schottmuelleri* infection of mice when activity of sulfisoxazole was determined in a constant, inactive amount of oleandomycin.

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Inactivation of Foot-and-Mouth Disease Virus by pH and Temperature Changes and by Formaldehyde. (23148)

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Methods of tissue culture for production of foot-and-mouth disease virus, type A, and for assay of the virus and its neutralizing antibodies were reported by Bachrach, Hess, and Callis(1). These methods have now been employed for measurements of stability of tissue culture virus under variable environmental conditions. The present report is concerned with rates of inactivation of virus by the action of hydrogen ion, heat, and formaldehyde.

Materials and methods. Virus. Foot-and-mouth disease virus, type A, strain 119 (FMDV-A119), was produced in Roux flask cultures of bovine kidney tissue(1). For preparation of the cultures, cut fragments of bovine kidney tissue were dispersed by incubation in 0.25% trypsin-buffer solution according to Youngner's modification(2) of the Dulbecco and Vogt(3) procedure for monkey kidney tissue. The liberated cells were decanted through 3 layers of gauze, centrifuged at low speed, washed twice in phosphate-buffered saline(3), and suspended at a 1:200 dilution in culture fluid. The fluid consisted of Hanks' salt solution containing 2% bovine serum and 0.5% lactalbumin hydrolyzate (Nutritional Biochemicals Corp.). Penicillin G, streptomycin sulfate, and phenol red were included at final concentrations of 100 units/ml, 0.1 mg/ml, and 0.005%, respectively. Roux flasks were each seeded with 75 ml of the cellular suspension and incubated at 37°C. Fluid changes were made after 3 and 6 days' incubation. By the second fluid change the cultures consisted of confluent outgrowths of epithelial and fibroblastic cells. The flasks containing the cultures were then inoculated with 4 ml of infective fluid from the preceding tissue culture passage and, after

being plugged with cotton, were incubated at 37°C. Infected culture fluids harvested 18 to 24 hours later contained as high as $10^{7.5}$ Reed and Muench(4) 50% infectivity doses per milliliter for bovine kidney cultures (TC ID_{50}/ml). These fluids after adjustment to pH 7.5 with dilute acid or alkali were sealed in ampules and stored at −40°C. When needed for experimental work the frozen virus was thawed to 4°C and clarified by centrifugation at 1,500 rpm for 10 minutes. *Kidney cell cultures in tubes were used for assaying virus.* In their preparation 0.4-ml volumes of cellular suspension prepared as described above were pipetted into culture tubes, 16 x 150 mm. The tubes were stoppered tightly and laid as close to horizontal as possible without wetting the stoppers. Incubation was carried out at 37°C, and fluid changes were made after 3, 6, and 8 days. The resulting outgrowths were suitable for use in the bioassay of virus between the sixth and the tenth day of growth. *For bioassay of virus,* serial 10-fold dilutions of the infectious samples to be tested were made in fresh culture fluid. Eight-tenth-ml volumes of the dilutions were inoculated into assay cultures, 5 cultures per dilution. After 48 hours' incubation, distribution of recognizable cytopathic changes was recorded, and TC ID_{50} values calculated. Cultures not showing these changes within 48 hours very rarely become infected upon further incubation.

Results. pH stability. The stability of FMDV-A119 was determined at pH values ranging from 2 to 10. Portions of thawed and clarified virus fluid were diluted 1:10 in cold veronal-acetate buffers of Michaelis(5) at pH 5, 6, 6.5, 7, 7.5, 8, 9, and 10. Additional virus samples were diluted in acidified saline at pH 2, 3, and 4. These acidified and buffered virus solutions were held in stoppered flasks at 4°C and were sampled periodically for virus assay. To avoid injuring cells of the assay cultures by adverse concentrations of hydrogen ions, the samples taken for assay were readjusted to pH 7.5 with dilute HCl or NaOH solutions as required. These adjustments were carried out with stirring to minimize development of high, local concentrations of the acid or base. In most cases buffer capacities were sufficient to hold pH values to within 0.05 of a unit of the nominal values over a period of several weeks. For continuous control, the pH value of each stored sample was determined at each sampling, and if found to deviate by more than 0.1 unit from the nominal value, a readjustment was made with dilute HCl or NaOH.

The inactivation curves for the 8 preparations in veronal-acetate buffer are shown in Fig. 1. The virus was most stable at pH 7 or 7.5, where its infectivity decreased only slightly over the entire 5-week period of observation. At pH 8 and 9 viral inactivations occurred at rates of approximately 90% per 3- and 1-week period, respectively. For samples stored at pH values 6.5 and 10, there was a 90% reduction in infectivity every 14 hours, and for samples at 5 and 6 a similar reduction was observed in less than 1 minute. Under conditions of this experiment the rates of inactivation of virus in the 3 acidified solutions at pH 2, 3, and 4 were too rapid to be measured.

Refinements in technics were required to determine more precisely the rapid rates of inactivation of acidified virus. A procedure was developed in which virus was instantaneously adjusted to the required pH value without subjecting it to conditions more acid than that desired. After being acidified for as short as 4 seconds, the virus was instantly neutralized without exposure to adverse alkaline conditions. A description of the technic, all steps of which were carried out at 4°C, follows. One part of the virus suspension was introduced carefully into the bottom of a test tube without contaminating the walls higher up in the tube. Nine parts of veronal-acetate buffer properly adjusted with respect to pH were poured into the virus, simultaneously agitating the tube to insure rapid mixing. To stop the reaction, an equal volume of neutralizing veronal-acetate buffer was poured into the virus-buffer mixture. The neutralizing buffer was adjusted beforehand to that alkalinity required to bring the acidified virus to pH 7.5. Virus was tested in this manner for its stability at pH 3, 4, 5, and 6.

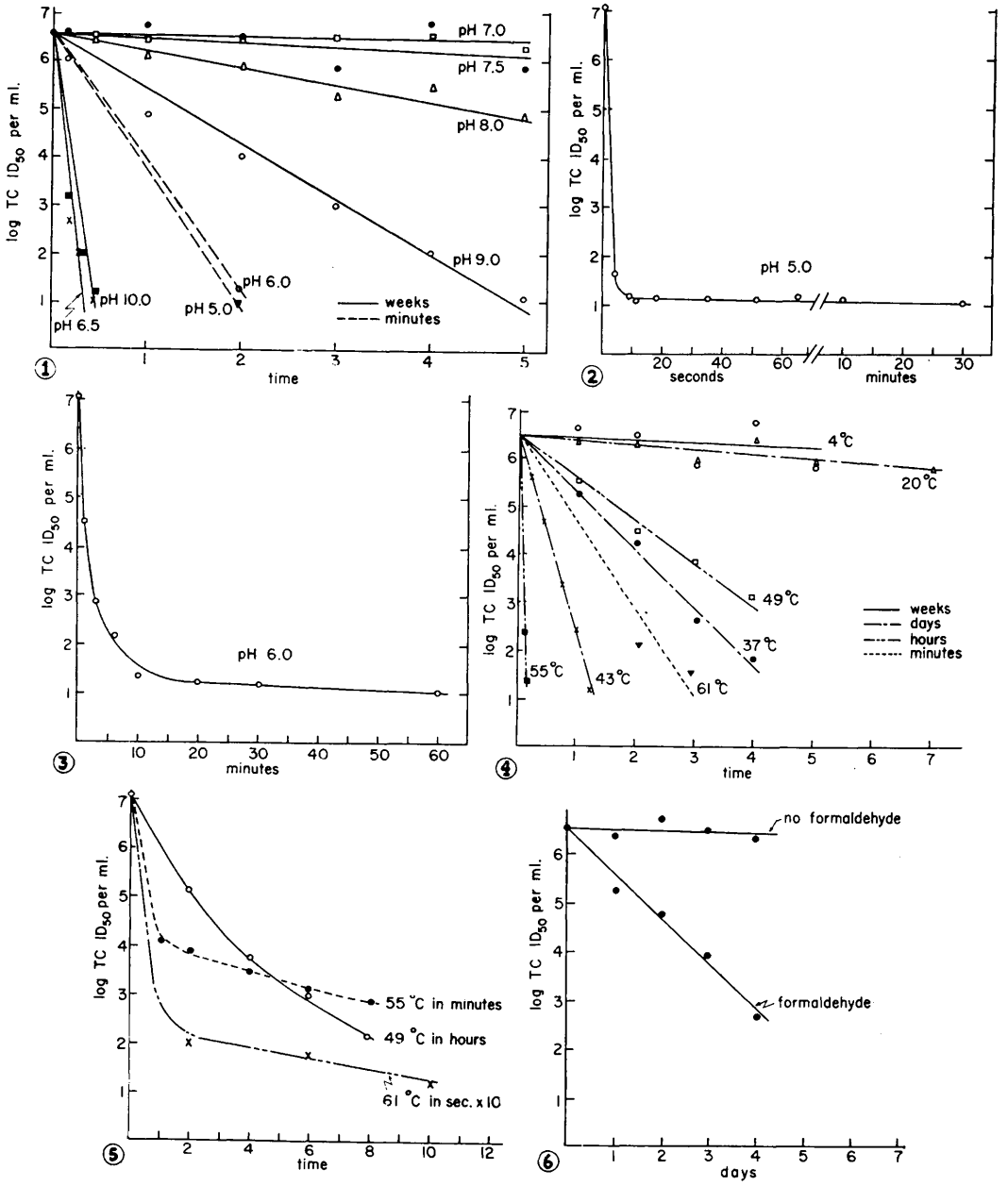

FIG. 1. pH stability of tissue culture foot-and-mouth disease virus, type A, at 4°C.

FIG. 2. Inactivation at pH 5.0 of tissue culture foot-and-mouth disease virus, type A, at 4°C. One-millionth part of the population remains infective for at least 30 min.

FIG. 3. Inactivation at pH 6.0 of tissue culture foot-and-mouth disease virus, type A, at 4°C, showing that about one-millionth of the population has a much greater stability than the rest.

FIG. 4. Thermal stability of tissue culture foot-and-mouth disease virus, type A, at pH 7.5.

FIG. 5. Inactivation at 49°, 55° and 61°C of tissue culture foot-and-mouth disease virus, type A, showing that at low survival levels the virus has increased heat stability.

FIG. 6. Inactivation of tissue culture foot-and-mouth disease virus, type A, by formaldehyde at a concentration of 0.009%.

It was found that veronal-acetate buffers adjusted to each of these pH levels brought the virus precisely to the nominal values. The pH values of the buffers required for neutralization were 11.60, 11.28, 8.70, and 8.15, respectively. Zero time values of infectivity were determined by titration of the original stock virus properly diluted in veronal-acetate buffer at pH 7.5.

Fig. 2 shows that virus was inactivated at pH 5 more rapidly than formerly depicted (Fig. 1), and in addition approximately one-millionth of the original virus population was resistant to further inactivation for at least 30 minutes. Fig. 3 shows that initial rate of inactivation at pH 6 was like that inferred in Fig. 1, but similar to the finding at pH 5, approximately one-millionth of the virus population was persistent to inactivation for a minimum of 60 minutes. This persistent fraction was present even in virus fluids which had been clarified at 10.000 rpm for 30 minutes before adjustment to pH 6. Inactivation of virus at pH 3 or 4 was so rapid that only one-millionth part of the infectivity remained after exposure for 10 seconds; after 45 seconds all infectivity detectable by tissue culture methods had disappeared.

Thermal inactivation rates of FMDV-A119 were determined from 4° to 61°C. A thawed and clarified virus suspension was diluted 1:10 in veronal-acetate buffer at pH 7.5. Portions of this suspension were placed in small Erlenmeyer flasks and brought rapidly to the temperatures shown in Fig. 4. At regular intervals samples were removed from each flask for virus assay. From the curves (Fig. 4) it was calculated that the time intervals required for thermal inactivation of 90% of the virus existing at any time were as follows: 0.5 minute at 61°, 2 minutes at 55°, 1 hour at 49°, 7 hours at 43°, 21 hours at 37°, 11 days at 20°, and 18 weeks at 4°C.

Additional experiments were devised which would permit a more exact study of the kinetics of rapid inactivation occurring at higher temperatures, namely, 49°, 55°, and 61°C. A procedure was employed in which virus suspensions at 20°C were raised instantly to the desired temperature and, after known intervals of exposure, were cooled instantly to 10°C. To accomplish this, one part of buffered virus at 20°C was mixed with 9 parts of veronal-acetate buffer held in a water bath at one-half degree higher than the temperature desired. An instantaneous adjustment of temperature was thereby effected. After a minimum interval of 15 seconds the reaction was stopped by diluting the heated virus sample with an equal volume of cold buffer. Typical results of several experiments are shown in Fig. 5. The results at 49°C were in close agreement with those found previously (Fig. 4) except for a slightly increased thermal stability of a small portion of the virus. At 55° and 61°C the inactivation rates were at least tenfold greater than those depicted earlier (Fig. 4) until survivals of 0.001 and 0.00001 were reached, respectively. (Survival refers to ratio of concentration of virus before and after exposure.) The virus fractions remaining after these survival levels were reached had greatly increased heat stabilities.

Formaldehyde inactivation. Inactivation of FMDV-A119 by formaldehyde at 4°C and pH 8 was determined. Frozen infected tissue culture fluid was thawed to 4°C, clarified, and then adjusted to pH 8 by dilution (1:10) in veronal-acetate buffer. Untreated portions of this fluid and portions treated with formaldehyde at a concentration of 0.009% were stored at 4°C and sampled daily for virus assay. Fig. 6 shows that formaldehyde-treated virus was inactivated at a rate of 90% per day. A similar result was found for virus which was adjusted to pH 8 with dilute NaOH, instead of buffer, prior to treatment with formaldehyde. At the time of formaldehyde experiments only the results involving short exposure at various pH levels were known (*i.e.*, Fig. 1, results through 3 days), and these did not indicate significant stability differences in the range of pH 7 to 8. Moreover, in the untreated control of the formaldehyde experiment (Fig. 6), there was little or no loss of infectivity due to exposure at pH 8 alone.

Discussion. The lability of FMDV-A119 of tissue culture origin under slightly acid

conditions is of particular interest. This behavior is in marked contrast to that of poliovirus, which is completely stable at a pH as low as 1.5 for at least 24 hours at 4°C(6). Such extreme differences in resistance of 2 animal viruses must reflect comparable differences in physical and chemical composition. The one-millionth part of the infective FMDV that resists inactivation at pH 5 and 6 disappears on lowering the pH to 4. Since virus populations clarified at 10,000 rpm for 30 minutes before acidification to pH 6 still contained a pH resistant fraction, the residual infectivity was probably not due to particles protected by aggregation. Nor were specific ions of the buffer involved since the controls in buffer at pH 7.5 did not lose infectivity. The residual infectivity was probably not due to reactivation of virus upon neutralization since there were no points of inflection in the pH curves. Studies are now in progress to determine if pH persistence is related to environmental conditions or genetic differences.

The importance of maintaining FMDV between pH 7 and 7.5 has already been observed in virus growth experiments carried out in Roux flask cultures of bovine kidney tissue. The pH in tightly stoppered cultures drops from 7.3 to 6.8 within a few hours after infection, with a concomitant decrease in the yield of virus. Once detected, this situation was corrected by providing for the escape of respiratory carbon dioxide by closing the flasks with cotton plugs instead of rubber stoppers at time of inoculation with virus.

The resistance of a small fraction of the virus to rapid inactivation at 55° and 61°C (Fig. 5) raises questions concerning the homogeneity of the population. Experiments are now under way to determine the basis of heat resistance and if it is related to pH persistence.

Activation energies for loss of infectivity have been derived from an Arrhenius plot(7) of thermal data (Fig. 7). The inactivation process can be described by first-order kinetics. The reaction rate constants were derived from the data of Fig. 4 except for the temperatures 49°, 55°, and 61°C, where the

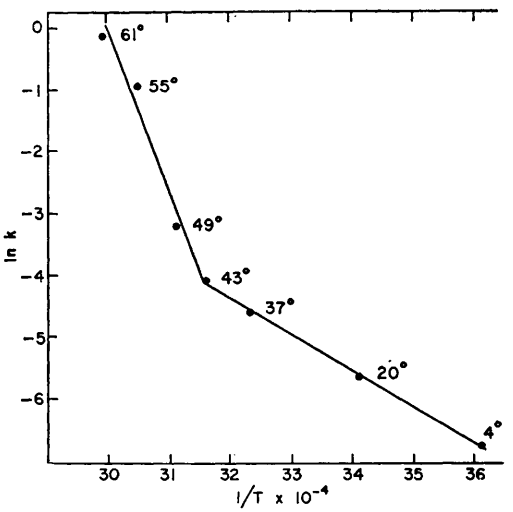

FIG. 7. Arrhenius plot of the thermal data for inactivation of foot-and-mouth disease virus. Logarithm of the rate constants for inactivation, k, *vs* the reciprocal of the absolute temperature, 1/T.

rates were determined from the initial slopes of the curves in Fig. 5. The Arrhenius plot gave 2 straight lines of different slope joining at about 43°C. Activation energies calculated from the slopes below and above 43°C were 27,200 and 120,600 calories per mole of FMDV, respectively. This result indicates that loss of infectivity by thermal action may have proceeded by 2 different processes. Speculating on the nature of such processes, Lauffer and Price(8) produced evidence at least with tobacco mosaic virus that high temperatures inactivated virus by denaturing the protein moiety faster than unknown thermal effects at lower temperatures.

Under conditions of temperature and pH approximately optimal for preservation of infectivity, FMDV was inactivated by formaldehyde according to first-order kinetics at a rate of about 90% per day (*i.e.*, close to that caused by exposure at 37°C alone). No part of the virus population appeared to possess above-normal resistance to formaldehyde. However, because of a lack of suitable technics, data for inactivations approaching zero infectivity were not experimentally obtainable. Thus, the time required for total inactivation of virus by formaldehyde is not necessarily determined by extrapolation of the slope in Fig. 6 to zero infectivity.

Summary. 1) Rates of inactivation of tissue-culture-derived, foot-and-mouth disease virus, type A, strain 119 (FMDV-A119), at various pH levels and temperatures and by formaldehyde were determined. Ranges of pH and temperature investigated were 2.0 through 10.0 and 4°C through 61°C, respectively: formaldehyde was employed at 0.009%. The results are interpretable by first-order kinetics. However, at pH 5 and 6 and also at 55° and 61°C small fractions of the virus population had much lower first-order inactivation rates than the bulk of the virus. Possibilities concerning the nature of the fractions with higher resistance are discussed. 2) Rates of inactivation at various pH levels were determined at 4°C. Below pH 4 the virus was totally destroyed within a few seconds. At pH 5 and 6 infectivity was lost at a rate of about 90% per second and minute, respectively, until only one-millionth of the virus remained. This residual virus was very stable to further inactivation. At pH 6.5 and 10, 90% of the virus was inactivated every 14 hours. The virus showed marked stability only at pH 7 and 7.5, losing little infectivity during a 5-week period. At pH 8 and 9, a 90% reduction of infectivity occurred within a 3- and a 1-week period, respectively. 3) Rates of thermal inactivation were determined at pH 7.5. The time intervals required for the inactivation of 90% of the virus existing at any time were as follows: 18 weeks at 4°; 11 days at 20°; 21 hours at 37°; 7 hours at 43°; 1 hour at 49°; 20 seconds at 55° to a survival of 0.001, 7 minutes thereafter; and 3 seconds at 61°C to a survival of 0.00001, 11 minutes thereafter. Activation energies calculated for loss of infectivity below and above 43°C were 27,200 and 120,600 calories per mole of FMDV, respectively. 4) Virus treated with formaldehyde at a concentration of 0.009% was inactivated at a rate of 90% per day of storage at 4°C.

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Weekly Variations in Serum Cholesterol Levels of Monkeys. (23149)

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Since the original reports of Peterson(1,2) on the hypocholesterolemic effect of soy sterols on cholesterol-fed chickens, many papers have appeared concerning the effect of soy sterols and b-sitosterol in lowering serum cholesterol levels in cholesterol-fed rabbits (3) and cholesterol-eating humans(4,5,6). The latter observation has not been confirmed in all instances(7). It has been suggested that the soy sterols exert their effect by interfering with the absorption of dietary cholesterol(4,8). In experiments using cholesterol-C^{14} interference was inferred by Chaikoff(9) and Kritchevsky(10) but not by Rosenman (11). The question of what influence soy sterols have on reabsorption of cholesterol has not been answered. As an effort in this direction we fed b-sitosterol to monkeys on a diet that was essentially cholesterol-free. Feedings were carried out daily for 6 weeks, with the animals being observed for two weeks before and 6 weeks after the feeding period. We observed no inordinate changes in the serum cholesterol levels either before or after the