Thermal and pH Stability of Adenovirus Types 12, 14 and 18. (29344)

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Adenoviruses have been reported to be stable under a variety of conditions including a wide pH range (Ginsberg, 1,2,3) Denny and Ginsberg(4); Pereira *et al*(5).

Hamparian *et al*(6) similarly found no decrease in the infectivity titer of types 3, 5, 7 and 12 at pH 3.0 after 30 minutes.

In the course of studies with adenovirus types 12, 14 and 18, it was, however, observed that virus harvests occasionally exhibited decreases in infectivity titer after long time intervals. This prompted an investigation into the inactivation kinetics of these types as a function of temperature and pH.

Methods and materials. Adenovirus types 12 (Huie strain), 14 (DeWitt strain) and 18 (D.C. strain) were obtained from the American Type Culture Collection and passaged 3 times in KB tissue culture. Overlay medium consisted of Eagle's Basal Medium (BME) in Earle's Balanced Salt Solution (BSS) containing 5% inactivated chicken serum. Penicillin and Streptomycin at 100 units and 100 μg/ml respectively were incorporated in the medium. Two or three days after complete cytopathogenicity, virus infected cells and fluid were freeze-thawed 3 times and centrifuged to remove cellular debris. The decanted supernatant fluid served as the virus pool* for all subsequent experiments.

Sodium phosphate buffers 0.2 M were prepared at pH 4.0, 6.0, 7.0, 8.0 and 10.0 (Beckman model G pH meter) utilizing appropriate mixtures of H_3PO_4, NaH_2PO_4, Na_2HPO_4 and Na_3PO_4. All buffers were sterile filtered prior to use through a Millipore† GS filter (0.22 μ).

For pH inactivation studies, virus fluids were diluted 1:5 in 0.2 M phosphate buffer and 1 ml dispensed into a number of 3 ml glass ampules. After varying intervals, ampules were removed and frozen at —70°C until titrated. All experiments were repeated at least once.

For freeze-thaw experiments, 10 ml of virus fluid previously freeze-thawed 3× (see above) were dispensed into vials and tightly stoppered. The contents were rapidly frozen in a dry ice-alcohol bath and thawed in a 37° water bath. Freeze-thaw cycles were repeated and 1 ml removed periodically and dispensed into vials for storage at —70° until titrated. Control samples were removed and frozen at —70° prior to beginning the freeze-thaw cycles.

Infectivity titrations were carried out in 24 hr old HeLa cultures planted at 50,000 cells per ml in BME with Earle's BSS (1.12 g $NaHCO_3$/l) containing 5% agamma calf serum. Titrations were performed with serial ten-fold dilutions of virus, 8 tubes per dilution, and 0.1 ml inoculated per tube. Hanks' BSS was used as diluent for all titrations. Cultures were refed after 3 or 4 days with 2 ml BME in Earle's BSS (2.2 g $NaHCO_3$/l) containing 5% agamma calf serum. The above media contained 100 units penicillin and polymyxin B sulfate‡ and 100 μg streptomycin per ml. A final reading was made at 7 days. Titers are expressed as $\log_{10}$ Tissue Culture Infective Doses—50% ($TCID_{50}$) per 0.1 ml calculated by the method of Reed and Muench(7).

Results. Thermal stability. When exposed to 56°C adenovirus types 12, 14 and 18 were rapidly inactivated. As illustrated in Table I, 3.4 and 4.3 logs of type 12 and 18 respectively were completely inactivated and 99.9% of type 14 was inactivated after 8 minutes exposure at 56°C.

Inactivation at 37° of types 12 and 14 proceeded exponentially with 95 to 99% virus inactivated after 10 days and 99.9% after 20 days (Table I). The pH of the virus

* These virus pools were obtained through the courtesy of Dr. Robert J. Huebner, Nat. Inst. Health.

† Millipore Filter Corp. Bedford, Mass.

‡ Burroughs Wellcome & Co., Inc., Tuckahoe, N.Y.

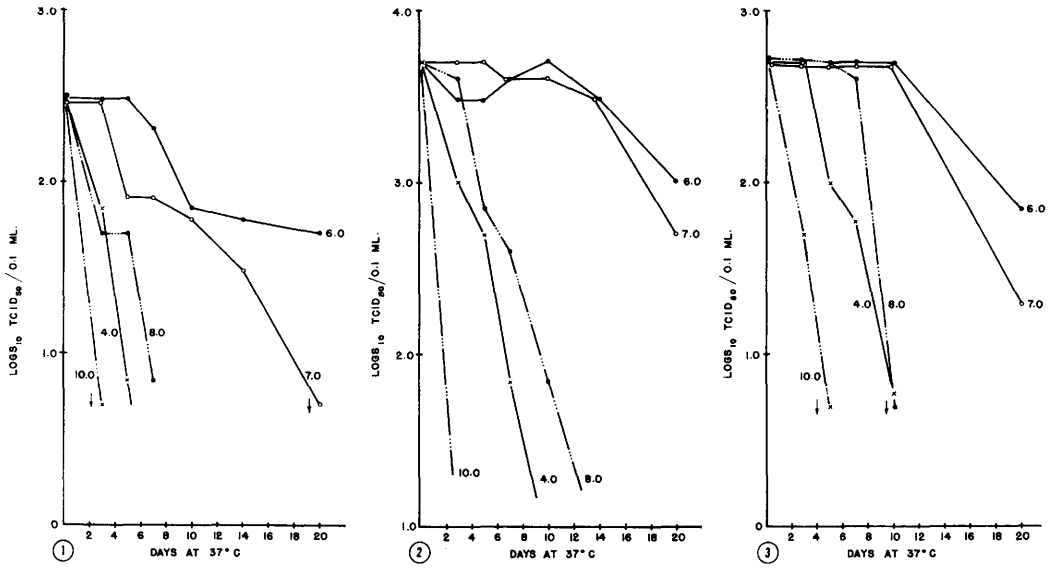

FIG. 1. Adenovirus type 12, 37°C inactivation kinetics at pH 4.0, 6.0, 7.0, 8.0 and 10.0.
FIG. 2. Adenovirus type 14, 37°C inactivation kinetics at pH 4.0, 6.0, 7.0, 8.0 and 10.0.
FIG. 3. Adenovirus type 18, 37°C inactivation kinetics at pH 4.0, 6.0, 7.0, 8.0 and 10.0.

fluid in these experiments was approximately 7.5.

Type 14, after 6 months and types 12 and 18 after 3 months at 4°C, exhibited no loss in infectivity.

Repeated freeze-thaw cycles. As seen in Table II, 30 successive freeze-thaw cycles caused no loss in infectivity of types 12 and 18. Type 14 similarly showed no inactivation after 15 cycles. It is apparent that under conditions of freezing and thawing as normally carried out in the laboratory, no loss in infectivity occurs.

TABLE I. Thermal Stability of Adenovirus Types 12, 14 and 18.

Adenovirus type	Infectivity titer ($logs_{10}$ $TCID_{50}$/0.1 ml) 4° months 0	3	6	37° days 0	10	20	56° minutes 0	4	8
12	3.5	3.5	—	3.1	1.7	.6	3.4	.6	neg
14	4.1	4.1	3.7	3.8	1.5	.5	4.3	2.5	.6
18	3.5	3.7	—	—	—	—	4.3	—	neg

Titrations performed in HeLa cell cultures. Serial 10-fold dilutions were used, 8 tubes per dilution, 0.1 ml inoculum per tube.

TABLE II. Effect of Repeated Freeze-Thawing on Adenovirus Types 12, 14 and 18.

Adenovirus type	Infectivity titer ($logs_{10}$ $TCID_{50}$/0.1 ml) Freeze-thaw cycles 0	5	10	15	20	25	30
12	3.4	—	3.4	—	3.0	2.5	3.2
14	4.5	4.5	4.5	4.5	—	—	—
18	3.0	—	3.5	—	3.2	3.5	3.5

Titrations performed in HeLa cell cultures. Serial 10-fold dilutions were used, 8 tubes per dilution, 0.1 ml inoculum per tube.

pH stability. Fig. 1, 2 and 3 illustrate inactivation kinetics at 37°C of types 12, 14 and 18 at pH 4.0, 6.0, 7.0, 8.0 and 10.0. It is apparent that all 3 types are maximally stable at approximately pH 6.0. At pH 4.0 and 8.0 there was almost complete inactivation in 10 days.

The slow rate of inactivation of type 12 at pH 6.0 does not proceed as a first-order reaction but, rather, occurs in 2 distinct steps (Fig. 1). After an initial 5-day period of stability, rapid inactivation occurs from 5 to 10 days, after which inactivation proceeds at a much slower rate. Interestingly, this 2-step inactivation curve is not observed at pH values other than 6.0, or with types 14 and 18.

Types 14 and 18 are somewhat more stable than type 12 (Fig. 2, 3). Type 18 in particular showed no demonstrable inactivation after 10 days at 37° at pH 6.0-7.0.

Discussion. Types 12, 14 and 18 were rapidly inactivated at 56°C, complete inactivation occurring with types 12 and 18 after only 8 minutes. These findings agree closely with those of Ginsberg(1) for types 1 to 4. Types 12, 14 and 18 show no decrease in titer after at least 3 months at 4°C. No loss in infectivity was evident with types 12 and 18 after 30 freeze-thaw cycles or type 14 after 15 cycles. These data attest to the stability of types 12, 14 and 18 and agree essentially with data reported by other investigators for other adenovirus types.

The pH inactivation kinetics at 37° under the above experimental conditions elucidate an important property of the adenoviruses studied, *viz.*, a marked increase in stability on the acid side of neutrality. Ginsberg(1) reported decreased stability at alkaline values for type 3; however, it was not determined if optimal stability was attained at acid pH, in particular, pH 6.0 as we have observed with types 12, 14 and 18. Denny and Ginsberg(4) observed only a slight loss in titer with type 14 after 7 days at pH 7.5. The results reported here are in agreement, although a marked loss occurs in 7 days at pH 8.0.

As ordinarily prepared, adenovirus harvests from cell cultures have a pH of 7.0 to 8.0; and if care is not taken to prevent loss of CO_2 the pH may be even higher. The present results show that there may be rapid loss of infectivity at these high pH values, and that the pH must be adjusted to 6.0 for maximum stability.

These findings indicate a need for a systematic examination of the pH stability of animal viruses. Hamparian *et al* (6) have shown that animal viruses fall into two groups on the basis of their stability at pH 3.0. Extension of these studies may reveal many more detailed systematic differences between classes of viruses with respect to pH stability.

Summary. Adenovirus types 12, 14 and 18 are relatively quite stable when exposed to 4° and 37°C. At 56°C types 12 and 18 are completely inactivated and type 14 more than 99% inactivated after 8 minutes. No inactivation occurs with type 14 after 15 freeze-thaw cycles, and types 12 and 18 after 30 cycles. The 3 types studied show maximal stability at pH 6.0. Infectious virus is rapidly inactivated on the alkaline side of neutrality. Types 14 and 18 demonstrate greater stability than type 12 at all pH values studied.

1. Ginsberg, H. S., PROC. SOC. EXP. BIOL. AND MED., 1956, v93, 48.
2. ———, *Ann. N. Y. Acad. Sci.,* 1957, v67, 383.
3. ———, *Virology,* 1962, v18, 312.
4. Denny, F. W., Ginsberg, H. S., *J. Immunol.,* 1961, v86, 567.
5. Pereira, H. G., Huebner, R. J., Ginsberg, H. S., Van Der Veen, J., *Virology,* 1963, v20, 613.
6. Hamparian, V. V., Hilleman, M. R., Ketler, A., PROC. SOC. EXP. BIOL. AND MED., 1963, v112, 1,040.
7. Reed, L. J., Muench, H., *Am. J. Hyg.,* 1938, v27, 493.

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Teratogenesis: Effects of Substituted Purines and the Influence of 4-Hydroxypyrazolopyrimidine in the Rat.* (29345)

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Certain substituted purines are recognized as potent teratogenic agents in experimental animals. For example, 6-mercaptopurine (6-MP) given to pregnant rats on the 12th day of pregnancy (62 mg/kg) produces

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