Characteristics of the New Respiratory Viruses (Adenoviruses*) II. Stability to Temperature and pH Alterations.† (22659)

HAROLD S. GINSBERG.
Departments of Preventive Medicine and Medicine, Western Reserve University, and University Hospitals, Cleveland, O.

To investigate the biologic characteristics of the respiratory viruses isolated independently by Rowe and co-workers(1) and by Hilleman and Werner(2) it was important to determine the stability of these agents under a variety of conditions. For practical considerations it was desirable to investigate the experimental conditions under which these viruses were inactivated, as well as the optimum and most practical conditions for handling and storing the agents. It is the purpose of this paper to present evidence that the 4 prototype viruses studied are relatively stable under varying conditions of pH and temperature.

Materials and methods. Tissue culture. Strain HeLa cells (Gey) derived from an epidermoid carcinoma of the uterus were employed(3). The methods used for the serial propagation of these cells and preparation of tube cultures were identical to those previously described(4,5). The nutrient fluid consisted of 40% human serum and 60% Hanks' balanced salt solution(3). *Viruses.* The viruses employed were types 1, 2, 3 and 4 of the new respiratory viruses(1,2) originally termed the Adenoid Degeneration or "AD" agents(1) or the RI-67 agents(2). More recently Huebner *et al.* have suggested the name adenoidal-pharyngeal-conjunctival (APC) viruses for this group of agents(6). A committee composed of interested investigators recently suggested the designation "Adenoviruses" for this group of agents(7), and this terminology will be employed hereafter. Types 1, 2, and 3 were kindly supplied by Dr. R. J. Huebner and type 4 (also termed RI-67(2) or ARD virus(4)) was furnished by Dr. M. R. Hilleman. The type designations are the same as those suggested by Huebner and co-workers (6). The viruses were propagated in HeLa cells using a maintenance mixture consisting of Scherer's MS 90%(3), and chicken serum 10% or MS 67.5%, chicken serum 7.5% and tryptose phosphate broth (Difco) 25%(5). Virus pools were prepared by freezing and thawing infected cells as previously described (5). Viruses were stored in sealed glass ampules at —70°C. *Infectivity titrations.* Serial 1:3.2 ($10^{-0.5}$) dilutions were prepared in Hanks' balanced salt solution, and 0.1 ml of each dilution was inoculated into each of 2 HeLa cell culture tubes. Inoculated tubes were incubated at 36°C for 6 days. A culture was considered infected when 50% or more of the HeLa cells had undergone specific cytopathogenic changes. The infectivity titer endpoint was calculated as the highest dilution of virus which infected one-half of the tubes inoculated. *Alterations of pH.* To adjust the viral suspensions to the desired pH, 0.5 N HCl or NaOH was employed. The quantity of HCl or NaOH required to obtain a desired pH was preliminarily determined using uninfected maintenance mixture. By this relatively crude method the number of drops of HCl or NaOH delivered by a calibrated capillary pipette in order to obtain a desired pH was placed in appropriate rubber stoppered tubes. To each tube was added 1.0 ml of virus infected maintenance mixture. After at least 5 minutes to permit complete mixture, the pH of each solution was determined electrophotometrically. The viral suspensions adjusted to the desired hydrogen ion concentrations were allowed to stand at room temperature for 30 minutes, after which the pH

* Name suggested to designate group of viruses previously termed AD, APC, or RI viruses(7).

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TABLE I. Storage of Adenoviruses at 4°C.

Virus	Virus infectivity titer after storage at 4°C (days) None	18	44	70
	(log)			
Type 1	−2.75	−2.75	−2.5	−2.75
2	−2.25	−2.5	−2.25	−2.25
3	−2.75	−3.0	−2.5	−2.75
4	−2.25	−1.75	−2.0	−2.0

TABLE II. Stability of Adenoviruses at Room Temperature (22-23°C).

Virus	Infectivity titer after storage at 22-23°C (days) None	2	4	7	10	14
	(log)					
Type 1	−3.5	−3.5	−3.5	−3.5	−3.5	*
2	−3.0	−3.5	−3.0	−3.0	−3.0	−2.5
3	−3.0	−3.0	−3.0	−3.0	−3.0	−3.5
4	−3.5	−3.5	−3.5	−3.5	−3.5	*

* Not tested.

of each was rapidly adjusted to pH 7.4-7.6 with 0.5 N HCl or NaOH. The total volume of NaOH and HCl which had been added to each tube had been recorded so that all viral samples, including the unaltered controls, were finally adjusted to the same volume with maintenance mixture. To determine viral inactivation, infectivity titrations were carried out on each aliquot in a given experiment at the same time. For one group of experiments a 0.1 M phosphate buffer at pH 6.5 was used to adjust the pH of the viral suspensions.

Results. Stability on storage. The 4 prototype viruses studied have been stored in the frozen state in glass sealed ampules at —70°C in a cabinet refrigerated with solid CO_2 and at —30°C in a mechanically refrigerated box for 19 and 7 months, respectively, the longest periods tested, without detectable decrease in infectivity titers.

It was next of considerable importance to determine whether the property of infectivity was stable at 4°C, ordinary refrigerator temperature. Pools of virus infected tissue culture homogenate were placed in screw-topped tubes at 4°C, and after 18, 44, and 70 days aliquots were removed and stored at —30°C until the completion of the test period. Infectivity titrations were done on all samples at the same time. The results of this experiment, summarized in Table I, indicate that these viruses can be stored at 4°C for over 2 months without loss of infectivity titer.

A similar experiment was carried out to determine whether the adenoviruses were inactivated when held at room temperature (from 22°-24°C) during the experimental period. The data from this experiment, presented in Table II, indicate that these viruses retained their infectivity titers at room temperature for 10 to 14 days.

Stability at 36°C. The above data indicated that the adenoviruses are relatively stable when stored over a wide range in temperature. The rate of inactivation of these agents when incubated at the temperature utilized for propagation of the viruses, *i.e.*, 36°C, was next determined. The results are summarized in Table III. For these experiments it was necessary to buffer the type 4 virus suspension in order to maintain a pH of 7.4. Preliminary experiments had shown that if the pH of type 4 virus preparations increased above 7.8 during incubation, the infectivity titer began to decrease within 24 to 48 hours; by 4 or 5 days the preparation would usually be non-infectious. As noted in Table III, however, when the pH of type 4 virus suspensions was maintained at a constant pH of 7.4 during incubation, this agent

TABLE III. Stability of Adenoviruses at 36°C.

Virus	Infectivity titer after incubation at 36°C (days) None	1	2	3	4	5	6	7
	(log)							
Type 1	−4.75	−4.75	−4.75	−4.0	−4.5	−4.5	−4.25 or >*	−4.25 or >*
2	−3.5	−3.75	−3.75	−3.5	−3.75	−3.25	−3.75	−3.5
3	−3.5	−3.25	−3.0	−3.25	−3.0	−3.0	−3.0	−3.0
4†	−3.25	−3.0	−3.0	−3.0	−3.0	−3.0	−2.5	−2.5

* Highest dilution tested, $10^{-4.0}$, infected both tubes.
† Buffered at pH 7.4 with 0.01 M phosphate buffer.

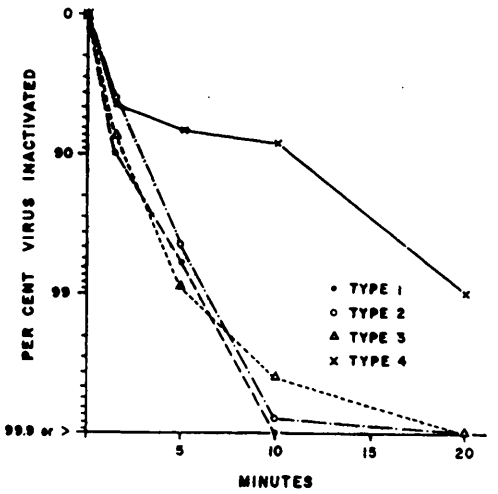

FIG. 1. Inactivation of adenoviruses at 50°C. Five 1.0 ml aliquots of each virus in tightly stoppered tubes were incubated in a 50°C water bath. At indicated intervals an aliquot of each virus was removed from the 50°C water bath and immediately immersed in an ice-water bath at 1°C. Infectivity titrations on all samples were done on the same day. Each endpoint represents geometric mean of 2 experiments.

was as stable as the other types. These data give further evidence of the marked stability of these agents, for when they were incubated at 36°C for as long as 7 days there was no measurable decrease of the infectivity titers by the methods employed.

Inactivation at 50°C and 56°C. To determine the rate and kinetics of the inactivation of the adenoviruses, the 4 agents under study were heated in 1.0 ml volumes at 50°C and 56°C for increasing periods. At the termination of the predetermined period of incubation the heated aliquot was immediately immersed in an ice-water bath at 1°C. Infectivity titrations on all samples of a single virus were done on the same day with a single lot of HeLa cell tubes. The results of 2 experiments with each virus are summarized in Fig. 1. Types 1, 2, and 3 viruses were inactivated at similar but not identical rates, whereas the type 4 virus was more resistant to inactivation at 50°C. It is of interest to note that the curves of inactivation of types 1 and 2 viruses could be interpreted to be straight line reactions. On the other hand, the curves of inactivation for types 3 and 4 viruses are not straight lines, which indicates that these agents were not inactivated at a uniform rate during successive unit time intervals at 50°C. No such differences in the rates of inactivation of these agents at 56°C were detectable, as all 4 viruses were more than 99.9% inactivated when incubated for only 2½ minutes and no residual infectivity could be detected after 5 minutes incubation at this temperature. That types 1-3 are noninfectious after incubation at 56°C for 30 minutes has been reported(6).

Degree of viral inactivation by varying pH. Multiplication of adenoviruses in HeLa cell cultures and the cytopathogenic alteration of the host cells induced by these agents is accompanied by an increase of lactic acid in the culture fluids(6,8,9) which may reduce the pH of the culture as low as pH 6.5 to 6.7(10). To determine whether the viruses may be inactivated during incubation of viral infected cultures the stability of adenoviruses was tested when the viral suspensions were adjusted to pH 6.5 and incubated at 36°C. These experiments clearly demonstrated that types 1-4 adenoviruses were not inactivated when incubated at 36°C for 24 hours at a pH of 6.5, conditions to which the viruses are often exposed during infection of HeLa cultures. To determine the stability of these agents under conditions which might be employed for chemical purification of viruses, as well as for other practical purposes, the inactivation of these agents over a wide pH range was also studied. The cumulative results of 2 or 3 experiments with each agent are summarized in Fig. 2. These data indicate that the adenoviruses are relatively stable at 22°-23°C between pH 6.0 and 10.0. It is of interest that type 4 virus was slightly less stable at acid pH's and type 3 virus was inactivated to a greater extent between pH 9 and 10 than were the other 3 prototype viruses studied. That these agents were not inactivated to a greater extent at pH's as low as 1.5-3.0 indicates that the infectious property is very stable under these unphysiologic conditions. Except for type 3 virus the agents under study were not inactivated to a significant degree between pH 7 and 10. At higher pH's, however, the viruses were readily inactivated,

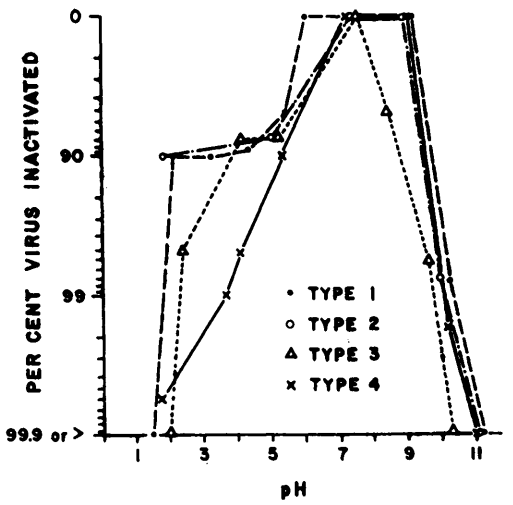

FIG. 2. Inactivation of adenoviruses by acid and alkaline pH changes. Adjustments of pH were made with 0.5 M NaOH or HCl. After pH change, all samples were held at 22-23°C for 30 min. pH of each aliquot was then adjusted to pH 7.4-7.6. Infectivity titrations on all aliquots in a single experiment were done on the same day. Each endpoint represents geometric mean of results from 2 or 3 experiments.

and at pH 11.0 infectious virus was not detected with any of the 4 types tested.

Discussion. The data presented indicate that the prototype viruses of types 1-4 of the new respiratory viruses(1,2), adenoviruses (7), are relatively resistant to inactivation both by heat and by pH changes. It must be emphasized, however, that only a single viral strain of each type was investigated; the variations of these characteristics within strains of a single type were not determined. Comparison of the types 1-4 viruses with the stability of other animal viruses indicates that their stability to heat and pH alterations is of the same order as the poliomyelitis(11-14) and Coxsackie viruses(15,16). All of these agents resist inactivation by ether(6,17-20). Further comparisons point out that the adenoviruses are considerably more stable to temperatures from 4°C to 50°C and pH alterations than are the influenza(21,22,23), mumps, and Newcastle disease viruses(24, 25). The stability of the adenoviruses when they are maintained at 4°C, room temperature (22°-23°C), or in the frozen state (—30°C to —70°C) facilitates attempts to isolate these agents from human sources. Furthermore, the capacity of these viruses to resist inactivation upon incubation at 36°-37°C eliminates a constant source of error from studies of the characteristics of the multiplication cycles(9,10) as well as other host-virus reactions(9,10).

The ecology of an infectious agent in many ways must be a reflection of the characteristics of the agent. That a virus can remain without inactivation of its important property of infectivity under a wide range of conditions should permit it to persist in nature either within or outside its host. These characteristics of stability similarly should permit the agents to spread from host to host under conditions which otherwise would make contagion difficult. Indeed, it may be considered that a basic understanding of the biologic and bio-physical characteristics of a virus not only facilitates insight into the activity of the agent in nature, but also permits hypotheses to be developed concerning the basic nature of the viral particle.

Summary. Types 1-4 of the new respiratory viruses, termed adenoviruses, were relatively stable when exposed to temperatures ranging from 4°C to 36°C. Type 1-3 viruses were inactivated in 10 to 20 minutes at 50°C, but the infectivity titer of type 4 virus was reduced less than 2 log units (99%) in 20 minutes. The adenoviruses were also resistant to inactivation at pH 6.0 to 9.0. The patterns of inactivation of the 4 viruses by alterations of pH as well as incubation at 50°C were similar, although consistent and significant differences among the 4 types were measured.

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Effect of Reduced Liver and Kidney Catalase Concentrations on Lethality of X Irradiation in Rats.* (22660)

WALLACE FRIEDBERG. (Introduced by Arthur C. Upton.)
With the technical assistance of Mary Elizabeth House.
Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.

Hydrogen peroxide, which is toxic to mammals(1), is a product of the X irradiation of water(2). The high percentage of water in the body, coupled with the presence of the hydrogen peroxide-reducing enzyme, catalase, in most living organisms(3) suggests a possible interrelation of X irradiation, hydrogen peroxide, and catalase.

According to Feinstein *et al.*(4), about 75% of the total body catalase in Sprague-Dawley rats is present in the liver and another 7% in the kidneys. Heim *et al.*(5) have reported that a single injection of 1 g of 3-amino-1,2,4-triazole kg of body weight in rats reduces the liver and kidney catalase concentration to about 11% of normal in several hours and that it takes more than 72 hr for the catalase to return to normal levels. It seemed of interest to determine whether reducing the liver and kidney catalase to low levels with this compound would increase the number of deaths in X-irradiated rats.

Materials and methods. Sprague-Dawley rats were used throughout. The 3-amino-1, 2,4-triazole (AT)† was used in concentrations of 50 mg/ml in water. Physiological saline (0.9%) was injected into all control animals. Both solutions were administered intraperitoneally in volumes of 20 ml/kg of body weight 4½ hr before either catalase assays or X irradiation. The results of the X-radiation experiments are shown in Table I. Inasmuch as the catalase-reducing effects of AT were reported for a different strain from that to those used in the experiments reported herein, the finding was checked. The liver catalase was assayed by the perborate method(6) and found to be depressed to 15-40% of that in

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