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Effect of Environmental pH on Adenovirus-Associated Virus (39085)

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(Introduced by D. M. Donaldson)

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The importance of controlling the environmental pH in cell cultures has long been recognized. Various cell functions have been shown to be influenced by pH, such as cell multiplication (3, 4), biochemical events (11, 13, 14), and cell mobility (17). The effects of pH on virus replication have been shown to be on a biochemical level during virus replication in cells infected with polio virus (12) and vesicular stomatitis virus (6). Synthesis of reovirus has also been reported to be pH dependent (5). It has been reported that recovery of latent parvoviruses from infected cell cultures is more efficient at pH 9 than at more physiological pH's (8). It has been reported that the effect of pH on H-1 parvovirus relates to the release of the virus from cell receptors rather than influencing the intermediate steps of virus replication (7).

The adenovirus-associated viruses (AAV) are defective parvoviruses which require coinfection with a helper adenovirus for complete replication. In the present study, we have found AAV particles to associate into increasingly large aggregates as the environmental pH is lowered. Because the usual bicarbonate buffered media show large fluctuations in pH, caution must be exercised in controlling the pH when infecting cells with AAV, or infectious titers and virus yields may be lowered, and the cells may not be infected at the effective multiplicity that is anticipated.

Materials and methods. Cells and media. Primary cultures of human embryonic kidney (HEK) cells and the HEp-2 cell line were obtained from Flow Laboratories, Inglewood, California. The KB cells were grown in suspension culture and infected with AAV as previously described (10). All media used were autoclavable media obtained from Flow Laboratories. The KB cells were grown in MEM modified for suspension culture, supplemented with 5% heat inactivated horse serum, various concentrations of sodium bicarbonate to effect pH changes, 100 units of penicillin per ml, 100 μg of streptomycin per ml, and 2 m*M* glutamine.

HEK and HEp-2 cells were grown in autoclavable MEM supplemented with various concentrations of sodium bicarbonate, 2 m*M* glutamine, 100 units of penicillin per ml, 100 μg of streptomycin per ml, and 10% fetal calf serum. HEK and HEp-2 cells were maintained on the above medium supplemented with 2% fetal calf serum. The horse serum, fetal calf serum, and antibiotics were obtained from Grand Island Biological Co., Santa Clara, California. The sodium bicarbonate solutions and HEPES buffer were obtained from Flow Laboratories.

Viruses. AAV type 1, strain H (AAV-1H) and AAV type 3, strain H (AAV-3H) used in this study have been previously described (2, 9, 15). The adenoid 6 strain of adenovirus type 2, used as helper virus, was previously described (16). Assays for infectious AAV were carried out by preinfecting cell monolayers in tubes with 10^5 median tissue culture infective doses ($TCID_{50}$) adenovirus type 2 helper, followed after a 2-hr incubation at 37° by dilutions of AAV. The tubes were incubated at 37° and read daily for the development of adenovirus cytopathic effects (CPE). At 4+ CPE the cultures were frozen, then assayed for AAV complement fixation (CF) antigen by the microtiter technique (18). The titer of the virus was taken as the reciprocal of the highest dilution showing positive AAV CF antigen.

Immunofluorescence assays. The presence of specific AAV antigens in infected HEp-2 cells was demonstrated by the indirect immunofluorescence test using AAV specific antiserum by techniques previously described (1).

Electron microscopy. Specimens of virus purified on isopycnic CsCl gradients were negatively stained on carbon stabilized, formvar-coated 200 mesh grids. The effect of

TABLE I. TITRATION OF AN AAV STOCK IN HEK CELLS AT VARIOUS pH'S

pH[a]	Sodium bicarbonate concentration (%)	Infectious virus titer ($TCID_{50}$/0.1 ml)	CF antigen[b] (units)
6.8	0.055	10^3	2
7.2	0.11	$10^{5.5}$	4
7.3	0.165	10^7	4
7.6	0.22	10^7	16
7.7	0.275	10^7	8
>7.7	0.33	10^7	8

[a] At time of AAV infection.

[b] Maximum units of AAV CF antigen in positive tubes.

pH on the aggregation of AAV particles was tested by mixing purified virus dialyzed in physiological saline in the absence of buffers, with equal volumes of 2% phosphotungstic acid (PTA) which had been adjusted to various pH's. The final pH was determined after mixing equal volumes of saline with the PTA preparations. The virus–PTA mixtures were incubated at room temperature for 15 min, then drops were placed on the grids for 2 min. The grids were then drained, dried, and examined in a Hitachi Hu 11 E electron microscope. The micrographs were taken at 75 KV at 18,000× magnification.

Results. Titration of an AAV-1 stock at various pH's. Dilutions of an AAV-1 stock were made in media at pH 7.6 containing 0.22% sodium bicarbonate. The infectious virus in these dilutions was assayed in HEK cells (Table I) and HEp-2 cells at various pH's. The pH was adjusted in the media with various concentrations of sodium bicarbonate from 0.055 percent to 0.33 percent. The pH's of the media at the time of AAV infection in the different series are recorded in this table. Infected cultures were identified by determining the induction of AAV CF antigen. The data in this table show that as the pH or sodium bicarbonate concentration was increased, more infectious virus was registered. Maximum titers of the stock were given at pH's above 7.3 in HEK cells. An increase in titer of four logs was noted in HEK cells between cultures infected at pH 6.8 and 7.3. The maximum titer of AAV CF antigen in the cultures was measured and showed an increase which correlated with the increase in infectious titer. Similar results were obtained in HEp-2 cells except the increase in titer as a function of pH was more gradual and the HEp-2 cells were not as sensitive in supporting AAV replication, showing an increase of 1.5 logs over this pH range.

Virus production in KB cells at various pH's. Parallel 250 ml suspension cultures of KB cells derived from a single 1000 ml culture were pH adjusted by adding increasing amounts of sodium bicarbonate. The cultures were preinfected with adenovirus type 2 for 6 hr at a multiplicity of three to five $TCID_{50}$ per cell. The cells were then superinfected with AAV-1 at about one $TCID_{50}$ per cell and incubation continued at 37° for 46 hr. At the conclusion of the incubation period, 50 ml portions of the cultures were removed for infectivity assays, and the cells from the remainder of each culture were harvested by centrifugation at 750*g* for 10 min and resuspended in 3 ml 0.01 *M* Tris buffer, pH 8.2. These cells were then freeze-thawed twice and assayed for CF antigen. Infectious virus assays were conducted in HEp-2 cells at an optimum pH. The results of this experiment showed that increasing the pH from 6.85 to 7.05 at the time of infection resulted in an 8- to 16-fold increase in CF antigen and a 10-fold increase in infectious virus.

Relationship of pH and sodium bicarbonate concentration to virus production. The effects on AAV multiplication presented above probably were either due to increasing sodium bicarbonate concentration or increasing pH. Experiments designed to distinguish between these two possibilities were conducted by buffering the medium with HEPES buffer at various pH's and supplementing the medium with various concentrations of sodium bicarbonate. These media were used in infectivity assays of an AAV stock. The results are shown in Table II. As the pH increased, the titers registered in the cultures increased regardless of whether the pH adjustment was due to increased sodium bicarbonate or addition of sodium hydroxide with no sodium bicarbonate in the medium. When the pH reached the 7.3 range optimum virus titers were detected; hence, the effect was a function of pH rather than of the sodium bicarbonate concentration.

TABLE II. TITRATION OF AN AAV STOCK IN HEp-2 CELLS IN HEPES BUFFERED MEDIA AT VARIOUS pH'S

HEPES (mM)	Sodium bicarbonate %	pH[a]	Infectious virus titer ($TCID_{50}$/0.1 ml)	CF antigen[b] (units)
25	—	6.55	$10^{1.5}$	1
25	—	6.7	$10^{1.5}$	1
25	—	6.8	$10^{1.5}$	<1
25	—	6.9	$10^{2.5}$	2
25	—	7.0	10^{3}	2
25	—	7.1	10^{4}	4
25	—	7.2	10^{4}	4
25	—	7.3	$10^{4.5}$	8
25 (pH 7.1)	0.055	7.2	$10^{4.5}$	8
25 (pH 7.1)	0.11	7.5	$10^{4.5}$	16
25 (pH 7.1)	0.165	7.7	$10^{4.5}$	4
25 (pH 7.1)	0.22	7.8	$10^{4.5}$	4

[a] At time of AAV infection.
[b] Maximum units of AAV CF antigen in positive tubes.

TABLE III. INDUCTION OF AAV IMMUNOFLUORESCENT ANTIGEN IN HEp-2 CELLS AT VARIOUS SODIUM BICARBONATE CONCENTRATIONS AND pH'S

Sodium bicarbonate (%)	HEPES (m*M*)	pH	AAV-1 Stain intensity (average)	AAV-1 Positive cells (% range)	AAV-3 Stain intensity (average)	AAV-3 Positive cells (% range)
0.055	—	varied[a]	2–3+	9–10	3+	16–17
0.11	—	varied	2–3+	10–11	1–2+	15–16
0.165	—	varied	±–1+	5–10	1–2+	5–9
0.22	—	varied	±–1+	5–10	<1+	2–3
0.275	—	varied	±–1+	4–5	±	2–3
0.33	—	varied	±	2–3	Neg.	0
—	25	7.1	1–2+	12–13		
0.055	25	7.3	1–2+	12–13		

[a] The plates containing the coverslips were placed in a CO_2 atmosphere after virus inoculation.

Induction of immunofluorescent (FA) antigen. The induction of AAV FA antigen was examined at pH's ranging from acidic to basic. Cover slips with monolayers of HEp-2 cells in petri dishes were infected with AAV-1 and AAV-3 following a 2 hr preinfection with Ad 2. The plates were incubated at 37° in a CO_2 atmosphere. The pH of each bicarbonate-buffered culture varied after removal from the CO_2 for inoculation and replacement in the CO_2, so accurate pH determinations could not be made. But over the series of cultures shown in Table III, the pH gradient ranged from acidic (phenol red indicator yellow) with 0.055% sodium bicarbonate to basic (phenol red indicator deep red) with 0.33% sodium bicarbonate. Whether or not sodium bicarbonate was required was tested in a culture infected in media buffered by 25 m*M* HEPES buffer at pH 7.1 and in a culture buffered by HEPES plus 0.055% sodium bicarbonate. These results are shown in Table III. It was somewhat surprising to find an apparent inverse relationship to that found for production of infectious virus and CF antigen. At acid pH's, the staining intensity of positive cells was greater than positive cells at higher pH's, and a markedly greater percentage of cells in the acid culture contained positive FA antigen. A similar decrease in FA antigen was noted in both AAV-1 and AAV-3 infected cultures. The decrease was related to increasing pH and appeared not to be enhanced or diminished by sodium bicarbonate. Optimal production of visible AAV FA

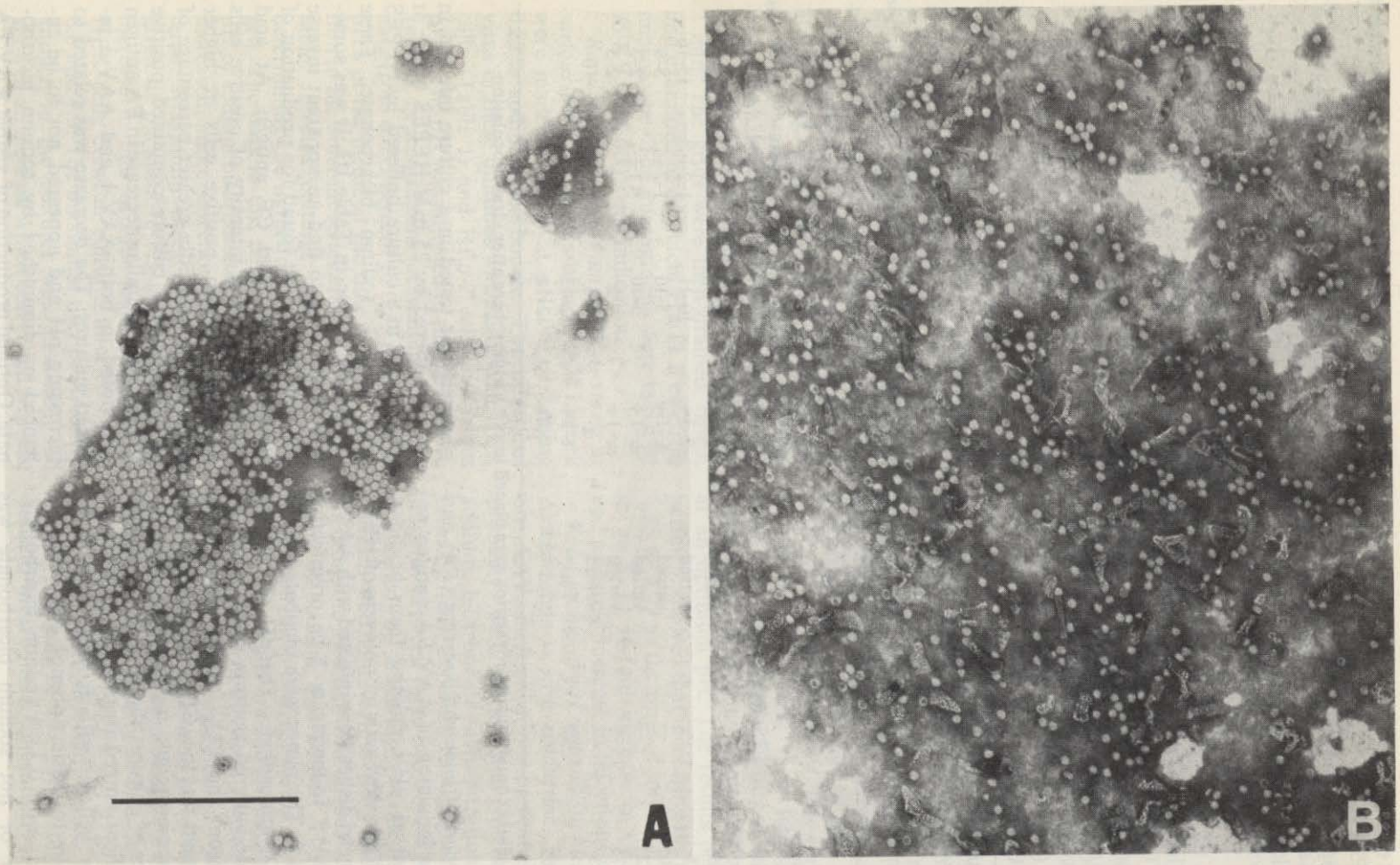

FIG. 1. Electron microscopic examination of AAV particles at different pH's. AAV particles were exposed to 1% phosphotungstic acid preparations adjusted to various pH's. Drops of each preparation were placed on carbon stabilized formvar-coated grids and examined in the EM. Aggregates were seen in all preparations at and below pH 7.2 (Fig. 1A). At pH 7.5 (Fig. 1B) the particles appeared singly, were evenly distributed, and no aggregates were seen. The bar represents a length of 500 nm.

antigen, therefore, was associated with acidic culture media.

Aggregation of AAV particles at various pH's. The effect of pH on the aggregation of AAV particles was examined by exposing purified AAV particles to preparations of PTA which had been pH adjusted from 2.9 to 7.5. Specimens of the virus in suspension were allowed to incubate in the PTA at the different pH's, and then were placed on grids and examined in the electron microscope for various degrees of aggregation. Representative electron micrographs of the results are shown in Fig. 1. At pH 7.5 (Fig. 1B), the virus particles occurred singly and were evenly distributed. At pH 7.2 (Fig. 1A) and all lower pH's tested, the particles were aggregated and were not evenly distributed in the field but were found in clumps, between which were large empty spaces. Many aggregates were found containing thousands of particles. At pH 7.2 the aggregates were reproducibly smaller than at pH 7.0 and below. Many free virions were observed in the background of the pH 7.2 preparations. Thus, aggregates of virus were present at pH 7.2 and below, but at pH 7.5 no aggregates were seen.

Discussion. The results of this study show AAV infectivity titrations, virus production, and induction of FA stainable antigen are all influenced by environmental pH. The greatest effect of pH appeared to be its influence on the aggregation of the viral particles. It should be noted that low multiplicities of infection were used throughout this study; hence, the effect of aggregation was not masked by high quantities of input virus. The question arose as to whether the effects were due to difference in pH or in the sodium bicarbonate concentration which was used for pH adjustments. The experiments using media buffered with HEPES buffer indicate that the major factor was pH and not sodium bicarbonate concentration.

The question of whether the pH affected primarily the helper advenovirus and only secondarily the AAV, was examined. At the lower pH values, development of adenovirus CPE was somewhat retarded (1–2 day lag behind optimal pH's) and there was consistently two- to four-fold more adenovirus CF antigen in cultures at optimal pH's. However, there was ample adenovirus present to support the replication of AAV because four plus adenovirus CPE always developed, and the adenovirus CF antigen titered consistently at 32 to 64 CF units in the acidic cultures. That the pH effects were not due to decreased cell activity at low pH's was indicated by the enhanced production of intracellular FA antigen at acid pH's and the ability of the cells to support adenovirus replication. Furthermore, the acid pH's studied appeared not to inactivate substantially the virus itself because of the AAV replication noted in the FA studies. The unexpected results of greater FA antigen levels seen in cells infected and maintained at acid pH's, raised the possibilities of greater antigen production in these positive cells or their decreased ability to release newly formed virus into the extracellular environment. We assayed the culture media from coverslip preparations for CF antigen and infectious virus and found virtually identical amounts of cell-released virus at all the pH's studied. (Note that in the other studies, showing less virus produced at acid pH's, the virus assayed was total infectious virus after freeze-thawing.) Since the released virus was identical in all cultures, it seems probable that at higher pH's more cells are producing less virus, and at acid pH's fewer cells are producing more antigen. This notion is consistent with the suggestion that at acid pH's the cells are infected with virus aggregates, resulting, in the time frame studied, in higher concentrations of translation products in positively staining cells, along with possible minimal contribution by input virus antigen. At the higher pH's where less viral aggregation is occurring, more cells become infected but fixation may terminate replication before sufficiently high concentrations of antigen accumulate for detection by immunofluorescence. Finally, we suggest that the effects of pH on AAV reported here are probably due to viral aggregation, indicated by the intercapsid associations occurring in the specimens prepared for electron microscopic examination.

Summary. The influence of environmental pH on AAV was studied in infectious virus titrations, induction of CF antigen, production of infectious virus, induction of

immunofluorescent stainable antigen, and aggregation of the viral particles. The pH of the medium was found to influence the titer of virus stocks in that less virus was registered at acid pH's, giving differences of up to 10^5 $TCID_{50}$ in HEK and HEp-2 cells. Less infectious virus was produced in KB cells, and decreased amounts of CF antigen appeared at acid pH's. However, increased levels of detectable intracellular FA antigen appeared at acid pH's. Electron microscopic examination of AAV particles negatively stained at various pH's showed increasingly large aggregates of particles as the pH was lowered. Under the acid conditions studied, the adenovirus helper and cell activities were only slightly suppressed, with the greatest effect due to aggregation of the virus particles.

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