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Conditions Affecting the Stability of a Simian Adenovirus in the Presence of Magnesium Chloride.* (31364)

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A number of enteroviruses have been shown to be protected from thermal inactivation by suspension in one or two molar concentrations of certain divalent cations(1,2,3). Contrariwise, the thermal inactivation of certain DNA viruses (adenovirus, herpes virus, pox virus, and papova virus groups)(4,5,6) and certain other RNA viruses (arbovirus and myxovirus groups)(5) has been shown to be enhanced in the presence of these concentrations of divalent cations.

In all of the above cited reports, the inactivation experiments were conducted using virus suspended in cell culture medium, either undiluted or diluted 10-fold in a serum-free, balanced salt solution. Such experiments fail to take into consideration the influence of medium components on either the enhancement of, or the stabilization against, thermal inactivation of virus infectivity in the presence of divalent cations.

This paper describes those conditions under which the thermal inactivation of a DNA virus, Simian adenovirus Type 15 (SV15), was not enhanced in the presence of 1M $MgCl_2$ at 50°C.

Materials and methods. The Simian adenovirus, SV15, used in these studies was obtained from Dr. J. L. Melnick and had been passaged 3 times in African green monkey kidney in his laboratory. The original source and previous passage history of this virus are unknown to us; however, we have reported(7) that this virus stock does not contain adeno-associated virus (AAV).

Monkey kidney cell cultures (MKCC) for virus assays were prepared according to the technique described by Wallis *et al*(8). Cultures were maintained in 0.5% lactalbumin hydrolysate (LA) in Earle's balanced salt solution (EBSS), which was supplemented with 0.5% agamma calf serum (Hyland).

Thermal inactivation studies at 50°C in 1M $MgCl_2$ were performed in the manner described by Wallis and Melnick(1). Virus titrations were done on the samples immediately after removal from heating. All virus dilutions (log_{10}) were made in LA medium free from serum and bicarbonate. Two-tenths ml from appropriate virus dilutions was inoculated into each of 4 tubes of MKCC, after which all inoculated tubes were placed in a roller drum and incubated at 36°C. Final readings for cytopathic effects (CPE) were made after incubation for 10-12 days.

Results. Effect of 1M $MgCl_2$ on SV15. Cell culture fluids containing SV15, as received from Dr. J. L. Melnick, were diluted 10-fold in serum and bicarbonate-free Hanks' balanced salt solution (HBSS). Portions of these diluted samples were further mixed with an equal amount of 2M $MgCl_2$ or deionized, distilled water (DDH_2O) and heated at 50°C. As can be seen from Table I, only slight inactivation of virus occurred (0.7 log_{10}) over the

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TABLE I. Survival of SV15* at 50°C in DDH_2O and 1M $MgCl_2$.†

Test agent	Minutes of incubation 0	30	60	90	120
DDH_2O	6.5‡	6.0	6.5	5.7	5.8
1M $MgCl_2$	6.3	4.0	3.0	2.3	1.0

* Simian adenovirus Type 15, passage 3 in Green Monkey Kidney cell cultures, as obtained from J. L. Melnick.

† Virus fluids were diluted 10-fold in HBSS and added to equal parts of DDH_2O and 2M $MgCl_2$.

‡ Log_{10} $TCID_{50}$ titer of virus per 0.1 ml.

2-hour period when suspended in DDH_2O; however a pronounced enhancement of virus inactivation occurred when SV15 was heated in 1M $MgCl_2$ (a 5.3 log_{10} loss in titer after 2 hours at 50°C).

Effect of dialysis of SV15 harvest fluids on inactivation by 1M $MgCl_2$. In a series of preliminary experiments, in which suspensions of SV15 were diluted 30- to 100-fold in HBSS prior to mixing with 2M $MgCl_2$, we failed to show any loss of SV15 infectivity when heated at 50°C over a period of 2 hours. These findings suggested that the inactivation of SV15 by 1M $MgCl_2$, observed previously, was enhanced by some factor present in the virus harvest fluids. To resolve the nature of this factor, the following experiment was done: 1) SV15 was inoculated into MKCC which were maintained in serum-free EBSS containing 0.2 g% $NaHCO_3$; 2) the virus was harvested by 5 cycles of alternate freezing and thawing after which particulate material was removed by low-speed centrifugation; and 3) 5 ml of the virus harvest fluid were dialyzed overnight at 4°C against 100 volumes of bicarbonate-free HBSS. Both dialyzed and non-dialyzed virus were then diluted 10-fold in HBSS and equal amounts of each diluted virus were added to a series of tubes containing DDH_2O or 2M $MgCl_2$. All tubes were then heated at 50°C for 2 hours.

Table II shows that heating dialyzed virus at 50°C in 1M $MgCl_2$ resulted in no detectable loss of virus infectivity, whereas non-dialyzed virus showed a progressive decrease in titer culminating in greater than a 4 log_{10} loss in the infective titer after 2 hours.

These results indicate that the enhanced loss of infective SV15 in 1M $MgCl_2$, observed with non-dialyzed virus, was influenced by a dialyzable component present in the virus harvest fluid.

Effect of pH on inactivation of SV15 by 1M $MgCl_2$. One of the more easily recognizable differences between the dialyzed and the non-dialyzed virus suspensions was the pH of the medium. Prior to diluting 10-fold in HBSS the pH of the dialyzed virus was 6.8, whereas the non-dialyzed virus had a pH of 7.8. After dilution in HBSS followed by addition to 2M $MgCl_2$ and heating for 2 hours, the pH of the dialyzed sample was 5.4-5.5 and of the non-dialyzed sample was 6.8. In view of these observations, a preliminary experiment was performed to determine the effect of pH on the enhancement of thermal inactivation of SV15 by 1M $MgCl_2$. The SV15 stock used in the previous experiment was dialyzed overnight at 4°C against 200 volumes of 0.9% NaCl, diluted 1:10 in DDH_2O, and further lutions of virus in the above test solutions 1) 0.05M Tris-buffer solutions which had been adjusted to pH 7.0 and to 5.2 with 0.1N HCl; 2) 0.9% NaCl, pH 6.0; and 3) DDH_2O. Four-tenths ml of the final dilutions of virus in the above test solutions was then added to each of a series of tubes containing an equal amount of pre-warmed (50°C) DDH_2O or 2M $MgCl_2$ and heated for 2 hours at 50°C.

The results shown in Table III indicate that the enhancement of SV15 inactivation by 1M $MgCl_2$ was a function of pH of the test material during the heating period, since in-

TABLE II. Survival of SV15* at 50°C in DDH_2O and 1M $MgCl_2$.† Effect of dialysis of the virus stock on inactivation by 1M $MgCl_2$.

Treatment	Test agent	Minutes incubation 0	30	60	90	120
Not dialyzed	DDH_2O	7.0§	ND‖	6.7	ND	6.7
	1M $MgCl_2$	6.7	5.5	4.5	3.5	2.3
Dialyzed‡	DDH_2O	6.5	ND	6.7	ND	6.7
	1M $MgCl_2$	6.7	6.7	6.7	6.5	6.5

* Virus stock prepared by inoculation into MKCC maintained in serum-free EBSS.

† Dialyzed and non-dialyzed virus suspensions were diluted 10-fold in HBSS and added to equal parts of DDH_2O and 2M $MgCl_2$.

‡ Dialyzed overnight at 4°C against 100 vol of bicarbonate-free HBSS.

§ Log_{10} $TCID_{50}$ titer per 0.1 ml.

‖ Not done.

TABLE III. Survival of SV15* at 50°C in DDH_2O and 1M $MgCl_2$. Dialyzed virus† diluted in Tris-buffer, NaCl‡ and DDH_2O prior to addition to the test agent.

Virus diluent§	Test agent	Minutes of incubation 0	30	60	120
Tris-buffer pH 7.0	DDH_2O	5.5‖	6.0	5.7	6.0
	1M $MgCl_2$	5.5	2.0	<.5	<.5
Tris-buffer pH 5.2	DDH_2O	5.5	5.5	6.3	6.0
	1M $MgCl_2$	6.0	5.7	6.2	6.0
.9% NaCl pH 6.0	DDH_2O	5.7	6.3	6.0	5.8
	1M $MgCl_2$	5.7	6.0	6.0	6.3
DDH_2O	DDH_2O	5.7	6.0	5.7	5.7
	1M $MgCl_2$	5.7	6.0	5.7	4.0

* Same virus stock as described in Table II.

† Dialyzed overnight at 4°C against 200 vol of 0.9% NaCl.

‡ Tris-buffer (0.05M) was adjusted to pH 7.0 and 5.2 with 0.1N HCl. 0.9% NaCl had a pH of 6.0.

§ Dialyzed virus was diluted 1:10 in DDH_2O and 1:10 in the described diluents prior to addition to equal amounts of DDH_2O and 2M $MgCl_2$.

‖ Log_{10} $TCID_{50}$ of virus per 0.1 ml.

activation of virus (greater than a 5 log_{10} loss in titer) occurred only in Tris-buffer at pH 7.0 in the presence of 1M $MgCl_2$ (and to a limited extent in water after 2 hours' heating).

To determine more precisely the pH conditions necessary for the inactivation of SV15 by $MgCl_2$, the following experiment was done. Dialyzed virus (from the previous experiment) was diluted 10-fold in distilled water and further diluted 10-fold in 0.05M L-histidine buffer adjusted to the pH values as listed in Table IV. Four-tenths ml of virus contained in the histidine buffer at the various pH's was added to each of several tubes containing pre-warmed 2M $MgCl_2$, after which all tubes were heated for 2 hours at 50°C. As can be seen in Table IV, no demonstrable loss of virus infectivity occurred in 1M $MgCl_2$ at pH 5.0 or 5.4 through 2 hours of heating. However, at pH 5.7 through 6.9 a progressive decrease in virus survival was demonstrated with increasing pH until, at pH 6.9, no infective virus could be recovered in undiluted test material.

TABLE IV. Survival of SV15* at 50°C in 1M $MgCl_2$. Effect of pH on inactivation by 1M $MgCl_2$.

Time of incubation	pH of diluent (.05M L-histidine) 5.0	5.4	5.7	6.0	6.4	6.9
0 hr	5.5†	6.0	6.3	6.0	5.7	5.5
2 "	5.5	5.5	4.5	3.0	2.3	<.5

* Same virus as was used in experiments recorded in Tables II and III. Virus was dialyzed overnight against 200 vol of 0.9% NaCl, diluted 1:10 in DDH_2O and then 1:10 in 0.05M L-histidine buffers at the various pH values listed in above table. After addition to an equal amount of 2M $MgCl_2$ and prior to heating, the pH dropped approximately 0.4 unit.

† Log_{10} $TCID_{50}$ of virus per 0.1 ml.

Discussion. In this report, we have demonstrated that the inactivation of a Simian adenovirus by 1M $MgCl_2$ was a function of the pH of the test medium containing the virus. Although marked inactivation of SV15 infectivity occurred when unaltered or undiluted cell culture fluids containing the virus were added to an equal amount of 2M $MgCl_2$, no inactivation could be demonstrated when these materials were tested after extensive dilution or after dialysis. Both dilution and dialysis would serve to eliminate the effect of the sodium bicarbonate, present in the culture fluids, on the pH of the test medium.

That sodium bicarbonate plays such a role in $MgCl_2$ experiments has been suggested by the data of Wallis and Melnick(9). They have reported that polioviruses were not stabilized by 1M $MgCl_2$ if vials, in which the virus-magnesium mixtures were contained, were left loosely capped. Under these conditions, the pH of the reaction mixture rose to 7.8 to 8.1, whereas the pH of these same mixtures in tightly stoppered vials was found to be 6.8 to 7.0.

Addition of an equal quantity of undiluted virus harvest fluids, or these fluids diluted 10-fold in HBSS, as was reported by Wallis and Melnick(1,2,4) has been found by us to result in a final pH well above that necessary for inactivation of SV15 by 1M $MgCl_2$. However, addition of an equal quantity of virus fluids containing little or no $NaHCO_3$ to 2 M $MgCl_2$, may result in a pH as low as 4.2, at which no inactivation of the SV15 occurs through the 2-hour heating period at 50°C.

In view of the present report, it becomes obvious that experiments such as the inactivation or stabilization of virus by divalent cations should be done under controlled pH conditions, since without such controls, these

experiments may be difficult to interpret and reproduce.

Our unexpected finding with SV15, as to its resistance to 1M $MgCl_2$, suggests the possibility that other DNA viruses, reported to be inactivated by 1M $MgCl_2$(4,5,6), may also demonstrate a similar dependence upon pH as a requirement for inactivation. These studies are now being carried out.

Summary. When Simian adenovirus Type 15 was heated at 50°C in the presence of 1M $MgCl_2$, it was found that inactivation of the virus infectivity was dependent upon the pH of the test medium. Dilution of dialyzed virus in 0.05M L-histidine buffer at pH 5.0 or 5.4 resulted in no inactivation of the virus following 2 hours' heating at 50°C in 1M $MgCl_2$; however, virus inactivation increased in response to an increase in pH from 5.7 to 6.9 under the same test conditions.

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Virus Particles in Hamster Tumors as Revealed by Electron Microscopy.* (31365)

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A group of 11 different hamster tumors, maintained by transplantation, were surveyed by thin section electron microscopy for virus particles. This group contained one tumor of spontaneous origin and some which arose after inoculation of viral agents into newborn hamsters of our closed colony. Two of the tumors arose in other colonies and were imported and maintained in a separate "open" colony. These are the Eddy fibrosarcoma induced by SV40 and the non-viral Fortner sarcoma No. 3B. The present report deals with preliminary positive findings of particles, some of which resemble murine leukemia agents and some of which appear identical with particles reported by Bernhard and Tournier(1) in tissue cultures of hamster cells and hamster tumors.

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Materials and methods. Pieces of fresh tumors were excised, minced in cold 8% glutaraldehyde and transferred to fresh glutaraldehyde for 1-2 hours. The tissues were next washed in phosphate-buffered saline (PBS) and post-fixed with 1% phosphate-buffered O_8O_4(2) for 1 hour. They were then dehydrated in graded alcohols, embedded in Araldite resin and thin-sectioned with a Porter-Blum MT-2 ultramicrotome equipped with glass knives. Sections were mounted on Parlodion-coated, carbonized grids and double-stained with uranyl acetate and lead citrate(3) for 5 minutes each. The uranyl acetate stain was prepared as a 2% aqueous solution with the pH adjusted to a point just short of precipitation (about 4.8) with 10 N NaOH.

Sections were examined in Siemens Elmiskop I or IA electron microscopes, and magnifications were calibrated with a grating