AN ALUMINUM MARKER FOR THE DIFFERENTIATION AND SEPARATION OF VIRULENT AND ATTENUATED POLIOVIRUSES*

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Several markers of poliovirus have been described which can be followed by tissue culture methods and which tend to discriminate between neurovirulent and attenuated strains.

Strains possessing the $d+$ marker grow more rapidly than $d-$ strains at low bicarbonate concentrations under agar (1, 2). Strains possessing the MS+ marker grow more readily on the monkey stable (MS) kidney cell line than MS− strains (3) and have a lower requirement for cystine (4). Strains possessing the T+ (or rct/40) marker grow readily at 40°C in contrast to the T− strains (5). The differential adsorption and elution of virulent and attenuated strains on cellulose columns (6, 7) or on aluminum hydroxide gel (8) have recently been described as other distinguishing characters. The intratypic antigenic marker recognizes that even within the same poliovirus type, strains differ in the rate at which they are neutralized by homologous or heterologous antisera (9).

From studies of the above characters of polioviruses and of their neurovirulence for monkeys and man, it has become evident that there is not available an *in vitro* method for reliably discriminating among strains according to their degree of attenuation (10, 11). However, strains with the above positive characters tend to have greater neurovirulence, and the well studied highly virulent strains are positive for all the markers. We have continued to search for additional genetic markers for polioviruses which may be related to virulence.

Enteroviruses, in contrast to adeno-, myxo-, herpes-, and poxviruses, have recently been found to be stabilized by divalent cations (12–14). In poliovirus harvests contaminated with vacuolating virus SV_{40}, the addition of divalent cations allowed ready destruction of the vacuolating virus and stabilization of the poliovirus (15). In extending these studies with cations we have discovered an *in vitro* marker which may be used to differentiate among poliovirus particles. Heating type 1 and 2 polioviruses at 50°C in $AlCl_3$ enhances the inactivation of virulent strains but stabilizes attenuated strains.

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This report is concerned with a description of the aluminum or A marker, its correlation with other *in vitro* markers, and its use to recover quantitatively attenuated type 1 and 2 strains from mixtures with virulent virus particles.

Materials and Methods

Monkey Kidney (MK) Cells.—Kidneys from immature rhesus monkeys were trypsinized, grown, and maintained in Melnick's lactalbumin medium as described in detail elsewhere (16, 17).

Viruses.—Virulent strains were plaque-purified lines of poliovirus type 1, Mahoney; type

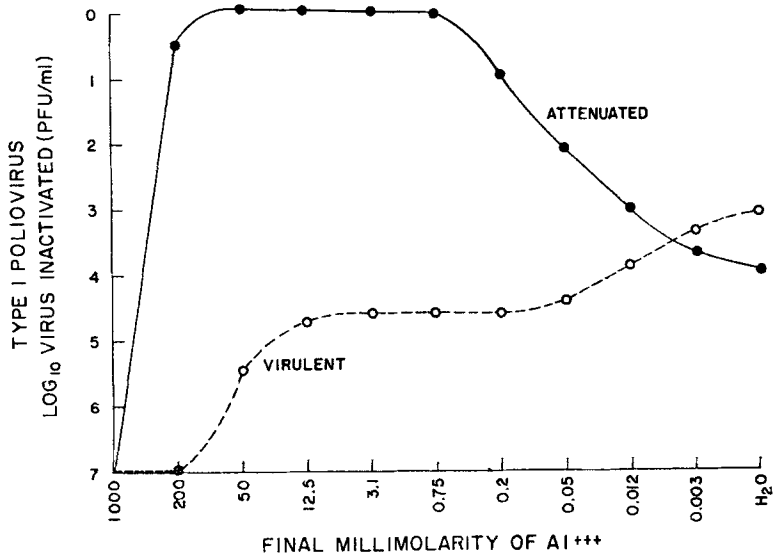

FIG. 1. The effect of Al^{+++} concentration on type 1 polioviruses heated at 50°C for 5 minutes. Virulent and attenuated strains were diluted to contain 10^7 PFU/ml in distilled water or in final concentrations of Al^{+++} designated.

2, MEF_1; and type 3, P24. Attenuated strains were Sabin's plaque-purified lines as used in the oral poliovaccine (type 1, LSc; type 2, P712; and type 3, Leon) (18). A number of strains were recovered from vaccinated children and tested for genetic markers (19).

Virus Assays.—All virus titrations were carried out by determination of plaque-forming units (PFU), using the bottle technique with the overlay medium described previously (12). $MgCl_2$ at a concentration of 25 mM was incorporated into agar to expedite plaque counting (20). The methods used in our laboratory for carrying out the *d* and T tests are described elsewhere (19).

Aluminum Ions.—$AlCl_3 \cdot 6H_2O$ was dissolved in sterile, distilled water to make a 2 M stock (48.3 gm per 100 ml), and distributed to a number of rubber-stoppered tubes, each of which was used only once. All concentrations of salts referred to in the text are final concentrations after addition to virus suspension. Similar results were obtained whenever an equivalent of $Al_2(SO_4)_3$ was substituted for $AlCl_3$. Serial dilution of virus-salt mixture for titration was made in physiologic saline (0.85 per cent NaCl).

Heating Methods.—Virus stocks were centrifuged lightly after thawing. The supernatant fluids were diluted tenfold in distilled water and mixed with an equal volume of $AlCl_3$ at twice the final concentration desired. Aliquots to be heated were delivered directly to the bottom of tubes and the top of each tube was flamed thoroughly to sterilize at least 1 inch of the uppermost surface of the tube. All test tubes used were uniform in size and thickness, measuring 13 × 100 mm, with an 0.8 mm wall. The tube was then rubber-stoppered and plunged into the water bath, covering the tube to ¼ inch from the stopper. At the end of the heating period, the tubes were quickly transferred into an ice water bath.

Toxicity of Al^{+++} for Cells.—Al^{+++} at concentrations below 0.04 M was not toxic for MK cells when 0.1 ml was inoculated into 1 oz. bottle cultures. When concentrations at this or higher concentrations were plated, the sample was first dialyzed to remove the salts. Incor-

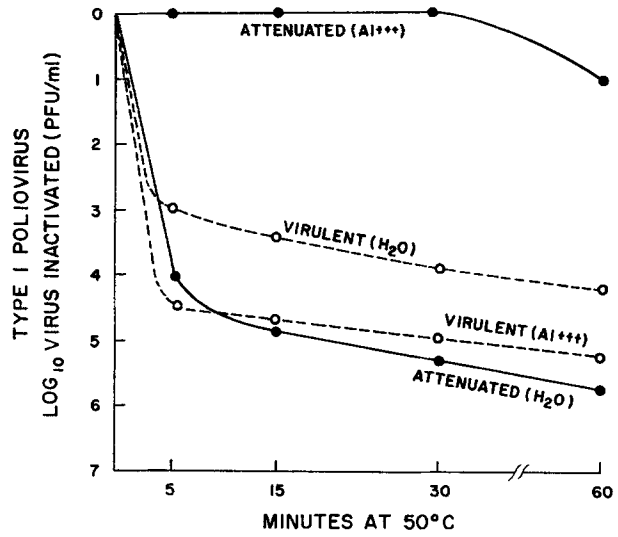

FIG. 2. Effect of heat on type 1 attenuated and virulent polioviruses, each present at 10^7 PFU/ml, in final concentration of 12.5 mM Al^{+++}.

poration of Al^{+++} in agar at concentrations below 0.02 M produced no inhibition of virus plaque number or size with any of the strains tested.

RESULTS

Stabilization of Type 1 Attenuated Poliovirus and Enhanced Inactivation of Type 1 Virulent Poliovirus in Al^{+++} at 50°C.—Type 1 attenuated and virulent polioviruses were diluted to contain 10^7 PFU/ml in H_2O and in different final concentrations of Al^{+++} as shown in Fig. 1. Samples were heated at 50°C for 5 minutes and then titrated. As illustrated in Fig. 1, Al^{+++} at high ionic concentration (1 M) enhanced inactivation of both strains. Control samples stored at room temperature (25°C) for 1 hour showed no detectable loss of infectivity in high or low concentrations of Al^{+++}. The type 1 attenuated strain was stabilized

at molarities from 0.05 mM to 200 mM with complete stabilization of the virus between 0.75 mM and 50 mM Al^{+++}. As the molarity of Al^{+++} was decreased below 0.75 mM, the infectivity was found to decrease accordingly, so that at 0.003 mM the results were the same as heating in water. On the other hand, the virulent strain tested simultaneously was inactivated at the same concentrations of Al^{+++} that stabilized the attenuated strain. More virus was inactivated in the Al^{+++} samples than in the controls heated in distilled water. At 200 mM Al^{+++} 7 $\log_{10}$ units of virulent virus were inactivated and between 0.05

TABLE I

Isolation of Attenuated LSc Virus after being Mixed with Virulent Mahoney Virus

	d test*			T test*		
	0.4 per cent $NaHCO_3$	0.07 per cent $NaHCO_3$	Marker	37°C	40°C	Marker
LSc						
Heated‡	6.1	<2.0	−	6.5	<1.0	−
Unheated§	6.3	<2.0	−	6.5	<1.0	−
Mahoney						
Heated	<2.0	<2.0		<1.0	<1.0	
Unheated	6.4	6.2	+	6.2	5.8	+
Mixture: LSc and Mahoney						
Heated	6.5	<2.0	−	6.2	<1.0	−
Unheated	6.3	6.2	+	6.2	5.8	+

* Titers are listed as PFU/ml for the *d* tests, and TCD_{50}/ml for the T tests.

‡ 15 minutes at 50°C in 12.5 mM Al^{+++}.

§ Held at 4°C in 12.5 mM Al^{+++} until titrated.

and 50 mM 4.7 $\log_{10}$ units were destroyed as against 3 $\log_{10}$ units in the sample heated in distilled water.

Using the same high concentration of virus, samples in distilled water and in 12.5 mM Al^{+++} were heated at 50°C for 0, 5, 15, 30, and 60 minutes. Fig. 2 shows the results of such an experiment. The Al^{+++} stabilized the attenuated strain completely for 30 minutes, and only 1 $\log_{10}$ unit was inactivated at 60 minutes. The control in distilled water lost 4 $\log_{10}$ units of infectivity after 5 minutes at 50°C and 5.7 $\log_{10}$ units at 60 minutes. The virulent strain in Al^{+++} was rapidly inactivated, 4.5 $\log_{10}$ units at 5 minutes and 5.2 $\log_{10}$ units at 60 minutes, even more rapidly than in water.

Type 1 attenuated poliovirus was diluted in distilled water to contain 10^7, 10^5, 10^3, and 10^2 PFU/ml in different final concentrations of Al^{+++} from 3 to 50 mM. Samples were heated at 50°C for 5 minutes, and inactivation curves for the virus suspensions at the 4 concentrations were similar to that shown in

Fig. 1. As the aluminum effect was present even after the virus had been first diluted 100,000-fold in water, there are no natural substances (proteins, amino acids, polysaccharides) in the infected cell cultures which play a role in the protection from thermal inactivation.

Separation of Attenuated Strains from Mixtures.—In view of the rapid inac-

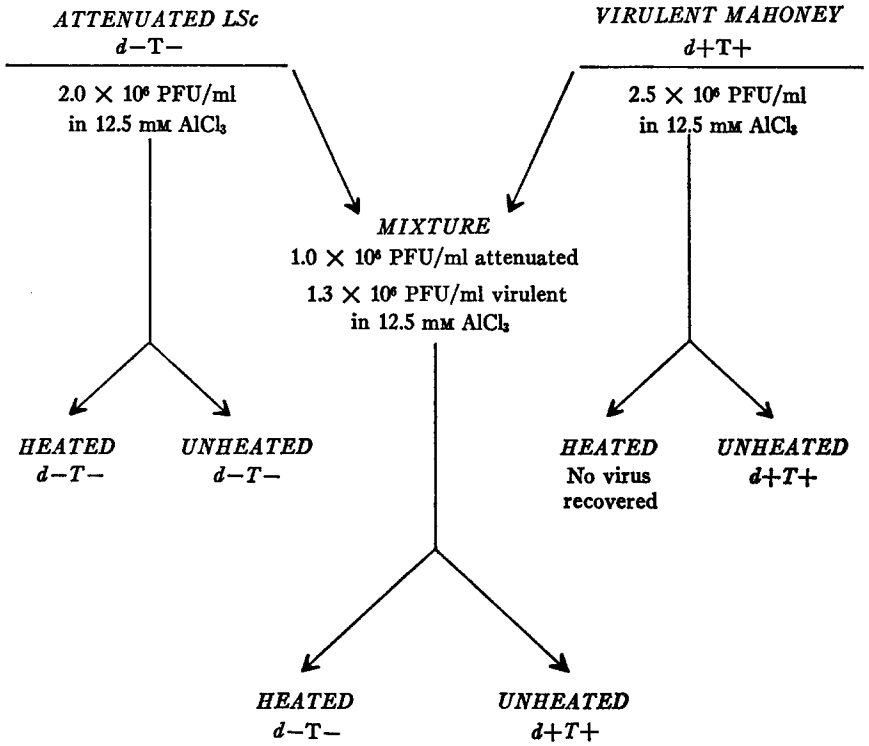

FIG. 3. Separation of type 1 attenuated and virulent polioviruses. The attenuated strain the virulent strain, and a mixture of both strains were heated at 50°C for 15 minutes in 12.5 mM Al^{+++}. Control (unheated) samples were also assayed. After heating only the attenuated *d*−T− strain survived.

tivation of type 1 virulent virus, and the stabilization achieved with the attenuated strain in aluminum solution, experiments were designed to determine if we could separate a mixture of these viruses. The attenuated and virulent strains were diluted to contain 2 × 10^6 PFU/ml in 12.5 mM Al^{+++}. One sample containing both viruses was made by mixing equal volumes so that the mixture contained 10^6 PFU/ml of each virus. Each sample was divided into two aliquots and one was heated at 50°C for 15 minutes. The results given in detail in Table I and illustrated in Fig. 3 show separation of the LSc strain from

a virulent-attenuated mixture. The attenuated virus (a $d-T-$ strain), showed no loss of infectivity after heating in Al^{+++}, and its d and T characters remained unchanged. The virulent virus (a $d+T+$ strain) was inactivated in the Al^{+++} sample heated for 15 minutes at 50°C, but the unheated sample in Al^{+++} was not altered in titer or in markers. For the mixture, the unheated sample yielded the $d+T+$ markers, for it is impossible to detect $d-T-$ particles when the proportion of virulent particles is as high as 50 per cent of the population. In contrast, the heated sample possessed $d-T-$ markers. In addition, measurement of plaque size was instructive. The two strains when mixed produced plaques of two sizes, 2 to 4 mm and 4 to 7 mm in size at 48 hours, representing attenuated and virulent viruses respectively. But, after heating in Al^{+++}, the plaques were of small size characteristic of the attenuated strain.

The A Marker Applied to Vaccine Progeny Strains.—Numerous type 1 strains recovered from subjects fed the oral vaccine containing Sabin's LSc strain were tested. Also, strains obtained from other sources were included, so as to cover strains with a different combination of d and T markers (*i.e.*, $d-T-$, $d+T-$, and $d+T+$).

The A test was carried out as follows: The viruses were diluted in 12.5 mM Al^{+++} to contain between 10^6 and 10^7 PFU/ml. Each virus was divided into two aliquots; one was heated at 50°C for 15 minutes, the other stored at 4°C until titrated.

Heated samples were assayed by using 0.1 ml of inoculum containing 200 to 300 PFU per bottle of original virus; unheated control samples were assayed using a tenfold higher dilution. Scoring was accomplished as follows: after 48 hours of incubation the control (unheated) samples should have counts of 10 to 30 PFU/culture. If so, heated samples with plaques too numerous to count were considered A− (no effect of heat). If the counts after heating ranged from 30 to 60, they were scored as A± (intermediate). When no plaques were evident or only an occasional plaque was seen (less than 10), the strain was scored A+ (inactivated by heat). The d and T marker scorings used in our laboratory have been described elsewhere (19). From the data on 37 strains in Table II it is evident that the A marker correlated with the d, but not the T marker. Of 10 A+ strains 9 were $d+$, only 2 were T+. Of 7 A± strains all were $d+$ and T−. Of 20 A− strains, all were $d-$ and T−. It is noteworthy that previous work has shown that $d-$ strains are almost always associated with T− strains.

Effect of Al^{+++} on Type 2 Polioviruses.—Type 2 attenuated and virulent polioviruses were each diluted to contain 10^7 PFU/ml, in distilled water and in different final concentrations of Al^{+++} as shown in Fig. 4. Samples were heated at 50°C for 5 minutes, and then assayed. As illustrated, the attenuated strain was stabilized from 200 mM to 0.2 mM Al^{+++}, and concentrations below 0.2 mM afforded no sparing effect over the virus heated in distilled water. Type 2 virulent strain reacted to heating in Al^{+++} quite differently than the type 1

virulent strain. The type 2 virulent virus was stabilized to some extent in Al^{+++} from 200 mM to 12.5 mM, but complete stabilization was not afforded at any concentration. Below the concentration of 0.75 mM and down to 0.12 mM this strain was inactivated to a greater degree than a heated sample in distilled water.

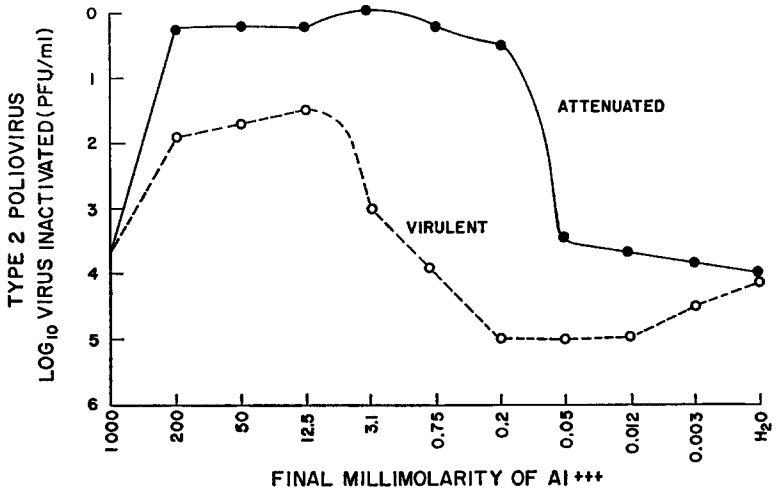

FIG. 4. The effect of Al^{+++} concentration on type 2 polioviruses heated at 50°C for 5 minutes. Virulent and attenuated strains were diluted to contain 10^7 PFU/ml in distilled water and in final concentrations of Al^{+++} designated.

TABLE II

Relationship of A Marker to d and T Markers for Type 1 Strains

	d + T +	*d* + T −	*d* − T −	Totals
A+	2	7	1	10
A±	0	7	0	7
A−	0	0	20	20
Totals	2	14	21	37

Using the same concentrations of virus described above, samples in distilled water and in 0.75 mM Al^{+++} were heated at 50°C for 0, 5, 15, 30, and 60 minutes. Fig. 5 shows the results of such an experiment. The attenuated strain was stabilized for 60 minutes in Al^{+++} whereas the control sample heated in distilled water was rapidly inactivated, 4 log units at 5 minutes, and 7 log units at 30 minutes. In Al^{+++} the virulent strain lost 3.3 log units at 5 minutes and 5 log units at 60 minutes, while in water it lost 3.7 and 7 log units at these periods.

To determine the value of Al^{+++} as a marker for type 2 strains, a number of specimens were tested as described for the type 1 viruses, but using 0.75 mM Al^{+++}. From the results on 20 strains shown in Table III it is evident that the A+ and A± strains were *d*+, while the A− strains were *d*−. As with the d

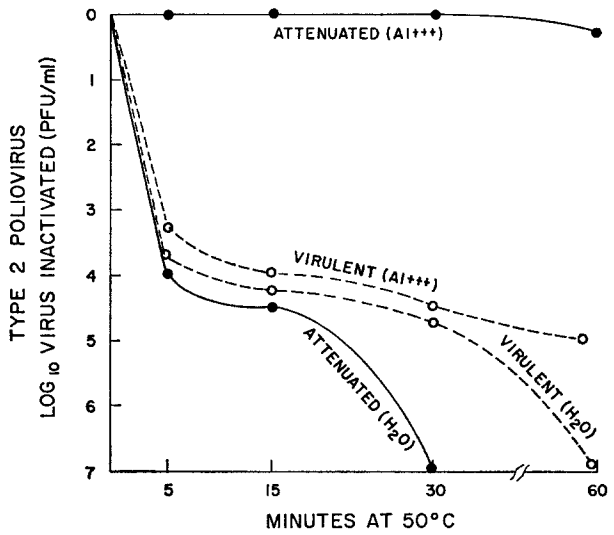

FIG. 5. Effect of heat on type 2 attenuated and virulent polioviruses, each present at 10^7 PFU/ml, in final concentration of 0.75 mM Al^{+++}.

TABLE III

Relationship of A Marker to d and T Markers for Type 2 Strains

	d + T +	*d* + T −	*d* − T −	Total
A+	2	1	0	3
A±	0	4	0	4
A−	0	0	13	13
Total	2	5	13	20

marker, the A+ and A± strains could possess the T+ or the T− marker, while the A− strains were all T−.

Separation of Type 2 Attenuated Strain from a Mixture with Type 1 Virulent Poliovirus.—Type 1 virulent and type 2 attenuated polioviruses were diluted so that each sample contained 5×10^4 PFU/ml in 10 mM Al^{+++}. An equal volume of each virus was mixed in one sample. The sample was divided into two aliquots and one was heated for 15 minutes at 50°C. The heated and

unheated aliquots were each divided into three samples, one sample mixed with an equal volume of type 1 antiserum, the second with type 2 antiserum, and the third with a mixture of types 1 and 2 antiserum. After 2 hours incubation at room temperature (25°C), each sample was titrated. As shown in Fig. 6, the unheated control aliquot in Al^{+++} treated with type 1 serum had a titer of 4.2×10^4 PFU/ml (type 2 virus); with type 2 serum, 5.1×10^4 PFU/ml (type

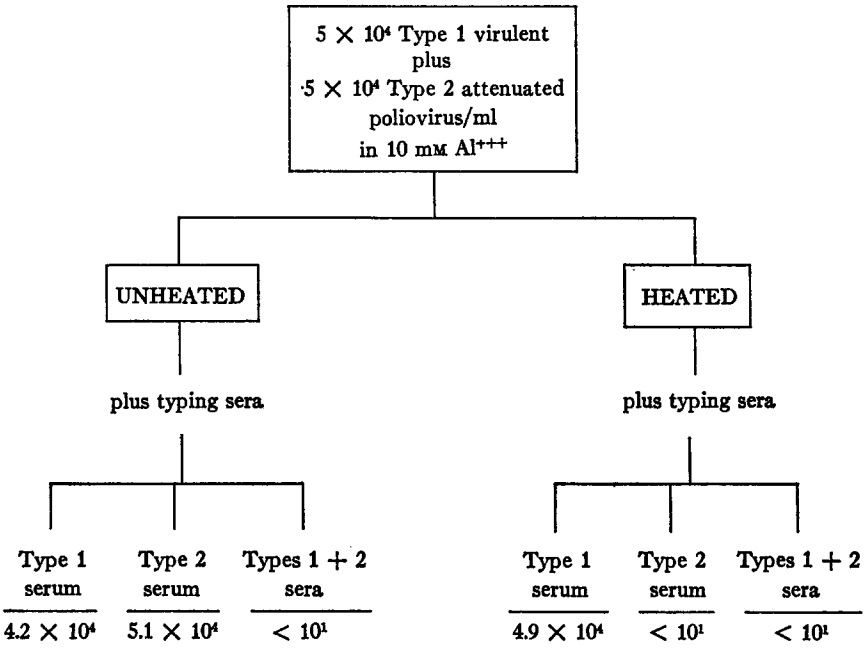

FIG. 6. Separation of type 2 attenuated strain from type 1 virulent poliovirus. A mixture of both strains was heated at 50°C for 15 minutes in 10 mM Al^{+++}. After heating, the sample was divided into three aliquots: one was treated with type 1 serum, a second with type 2 serum, and the third with a pool of types 1 and 2 sera. The control (unheated) sample was treated similarly, and all specimens assayed for virus. Only the type 2 strain was recovered after heating.

1 virus); with the combined types 1 and 2 sera there was no detectable infectivity, as both viruses were blocked. The heated sample treated with type 1 serum showed a titer of 4.9×10^4 PFU/ml, representing the type 2 virus resisting inactivation in Al^{+++} at 50°C. The heated sample treated with type 2 serum yielded no detectable infectivity, indicating neutralization of the type 2 virus by homologous serum and *inactivation* of type 1 virus by heating in Al^{+++}.

Effect of Al^{+++} on Type 3 Polioviruses.—The results of similar experiments with the attenuated and virulent type 3 strains are shown in Figs. 7 and 8. The attenuated strain was stabilized from 0.2 to 12.5 mM Al^{+++} with the optimum

sparing effect at 3 to 12.5 mM. The virulent strain manifested some sparing effect at all molarities with optimum effects at 12.5 to 50 mM. Contrary to the results with other virulent strains of the other types, no enhanced inactivation

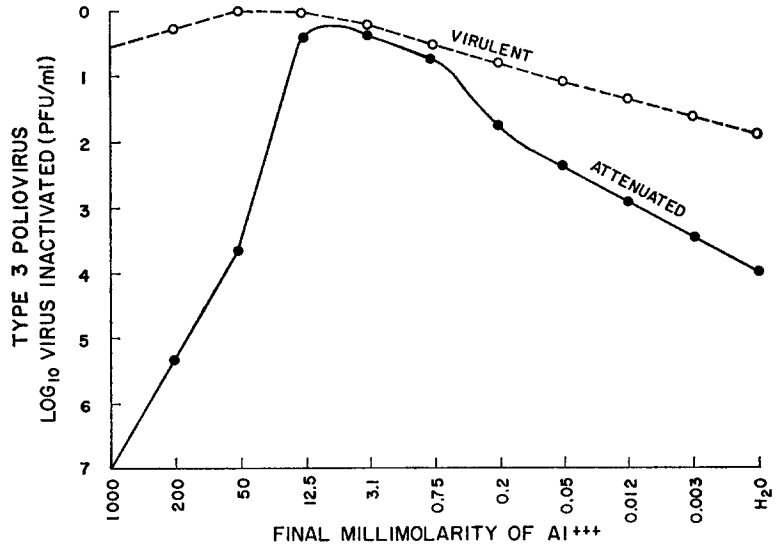

FIG. 7. The effect of Al^{+++} concentration on type 3 polioviruses heated at 50°C for 5 minutes. Virulent and attenuated strains were diluted to contain 10^7 PFU/ml in distilled water or in final concentrations of Al^{+++} designated.

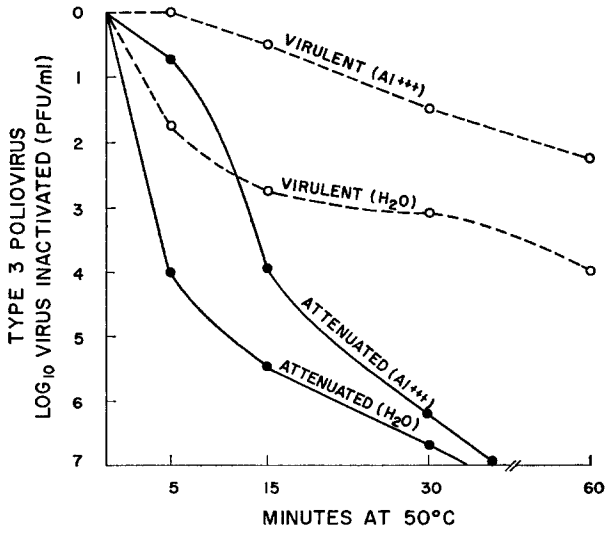

FIG. 8. Effect of heat on type 3 attenuated and virulent polioviruses, each present at 10^7 PFU/ml, in final concentration of 10 mM Al^{+++}.

occurred with the type 3 strain. Because the pattern diffused from those with the type 1 and type 2 strains, the A marker could not be applied in the same way to the vaccine progeny strains.

DISCUSSION

Another characteristic for detecting differences between virulent and attenuated poliovirus has been described in this report; it is called the A marker. Al^{+++} in low concentrations protects attenuated type 1 and 2 strains from heat inactivation but enhances the thermal inactivation of virulent types 1 and 2 strains. When heated at 50°C for 15 minutes, the optimal Al^{+++} concentration for detecting these differences varied from 12.5 mM for type 1 strains to 0.75 mM for type 2 strains. Type 3 strains showed opposite, but less marked, differences in thermolability in the presence of Al^{+++}. In recent studies on the enhancement of cell susceptibility to poliovirus by Mg^{++}, the type 3 attenuated and virulent strains also behaved differently from those of types 1 and 2 (20). Woods and Robbins (8) also found that as regards the elution from $Al(OH)_3$ marked differences existed for virulent and attenuated type 1 strains, which were less for type 2 strains, but which were hardly detectable for type 3 strains.

It is noteworthy that the A marker correlates with the *d* character, especially for type 1 and 2 strains. Thus strains of these types which grow rapidly at low bicarbonate concentrations are those most readily inactivated by heat in the presence of Al^{+++}, and conversely those strains which grow poorly under low bicarbonate are stabilized by Al^{+++} to thermal inactivation. There was no correlation with the reproductive capacity at 40°C (rct/40 or T marker) of a strain. As documented elsewhere (1, 10, 19, 21) strains which are positive for the *d*, and particularly those positive for both the *d* and T markers, are more apt to possess greater neurovirulence than negative strains.

In view of the findings reported here, the A marker may also be used as a test for the genetic stability of vaccine strains after propagation in man. The A test has a decided advantage over the *d* test in the ease with which it is carried out. No CO_2 incubator is required, and the results are not complicated by having to distinguish between normal and delayed plaques. In the A marker test, one measures all the viable viruses present. If the approximate titer of the strain is known, the test can be set up using 2 bottle cultures for the unheated sample and 2 bottles at ten times the virus dose for the heated sample.

The fact that Al^{+++} stabilizes the types 1 and 2 attenuated rather than the virulent strains has allowed us readily to separate the attenuated strain from mixtures containing a high proportion of virulent poliovirus particles of the same or of another type. This suggests three applications: (1) maintenance of the purity of an attenuated strain by subjecting each virus harvest to heat treatment in the presence of Al^{+++} to destroy any virulent mutants which might have arisen in the propagation cycles, or any virulent contaminants

which might have entered from outside sources; (2) selection of attenuated strains as vaccine candidates from natural mixtures or laboratory–manipulated viruses, and (3) counting and selecting attenuated progeny in genetic experiments.

SUMMARY

A new character, the *A* marker, for polioviruses is described. In the presence of Al^{+++} (1 to 100 mM) attenuated, but not virulent, strains of type 1 and type 2 polioviruses are stabilized so that they resist thermal inactivation at 50°C. Differences between the attenuated and virulent type 3 strains studied were of the opposite character and less marked.

By the use of optimal concentrations of Al^{+++} (12 mM for type 1 and 1 mM for type 2) attenuated strains can be separated from mixtures containing virulent type 1 or 2 strains.

The A marker was found to be correlated with the *d* but not the rct/40 or T marker. The A marker has been used for following genetic changes of vaccine strains after their multiplication in man.

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