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Extraordinary Effects of Specific Monovalent Cations on Activation of Reovirus Transcriptase by Chymotrypsin In Vitro

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Activation of reovirus transcriptase activity, latent in intact virions, by digestion of purified virions with chymotrypsin (CHT) in vitro shows a stringent requirement for specific monovalent cations. Cs^+, Rb^+, or K^+ ions are capable of facilitating activation by chymotryptic digestion. Na^+, Li^+, or NH_4^+ ions are not capable of facilitating the CHT activation of polymerase activity and are antagonistic towards the effects of the facilitating ions. The data indicate that the effect of the cations is exerted on activation of the polymerase activity by CHT as opposed to an effect on polymerization per se. This effect may be important biologically in that it provides a mechanism whereby the virion can sense whether it is in an intracellular or an extracellular environment and thereby can avoid premature uncoating.

Reovirus, an animal virus, possesses a genome composed of 10 segments of double-stranded ribonucleic acid (dsRNA) encapsidated within a double layer of protein capsomers (11, 16). During the normal infectious cycle, some of the proteins which constitute the outer capsid are removed, and the virions are thereby converted to subviral particles (SVP) (5, 18) which contain the endogenous transcriptase activity associated with reovirions. These SVP then serve as the structural and functional units of infection, actively synthesizing viral messenger RNA and eventually leading to replication of the viral genome and to the production of progeny virus (1, 5, 17, 18).

Virions of reovirus can be converted to cores in vitro by digestion with chymotrypsin (CHT) under appropriate conditions. The in vitro cores, as reported by several workers (5, 10, 14, 17, 20), possess RNA polymerase activity in vitro similar to that of SVP from infected cells, although the in vivo and in vitro particles are not identical by a variety of criteria. Recently we have been interested in studying the properties of the transcriptase activity in vitro with special emphasis on the possibility of demonstrating regulation of transcription similar to that demonstrated to be operating in vivo (8, 12). Because it is comparatively well defined, the reovirus SVP transcription complex may be a potentially useful model system for elucidating mechanisms of transcription control operating within eukaryotic cells. During the course of our studies, we consistently noted that the transcriptase activity of extensively purified virus preparations which had been exhaustively dialyzed against 0.1 M tris(hydroxymethyl)aminomethane-chloride (Tris-chloride), *p*H 8.3, containing 0.1 M NaCl could not be activated by digestion with CHT, although temperature shock activation was very effective. Detailed investigation of this phenomenon has revealed a remarkable requirement for specific monovalent cations for activation of the reovirus transcriptase by CHT digestion in vitro. In brief, we have found that Cs^+, Rb^+, or K^+ ions are required to permit activation of reovirus transcriptase activity by CHT. Na^+, Li^+, or NH_4^+ ions are not capable of facilitating this activation and are antagonistic towards the effects of the facilitating ions. In this communication we describe detailed experiments revealing these ion-reovirion-CHT interactions.

MATERIALS AND METHODS

Cells and virus. Reovirus type 3 Abney strain was obtained from the American Type Culture Collection. This was adapted to growth in mouse L cells by repeated passage on monolayer cell cultures. After plaque purification, stocks for routine use in experiments were grown up in suspension cultures of L

cells. Mouse L cells were routinely grown as suspension cultures in minimum essential medium (MEM) with spinner salts (F14; supplied by GIBCO, Grand Island, N. Y.) supplemented with 7% fetal bovine serum (FBS) (BBL, Division of Becton, Dickinson & Company, Clarkson, Ontario). Spinners were maintained at 37 C in a water bath. Under these conditions cells in exponential-growth phase had a doubling time of 16 to 18 hr.

For infection, exponentially growing cells were collected by centrifugation (10 min at 500 × *g*) and resuspended at approximately 10^7 cells/ml in fresh MEM without serum. Supernatant fluid was saved for use as conditioned growth medium. Reovirus cell lysate which had been frozen and thawed through three cycles was added to the resuspended cells to obtain a multiplicity of infection of between 5 and 10 plaque-forming units/cell. The virus was allowed to adsorb for approximately 1 hr at room temperature, with the cells kept in suspension throughout this time. The infected cells were then diluted to a concentration of 5 to 10 × 10^5 cells/ml with MEM plus 7% FBS, and the spinners were transferred to 37 C (this was taken as zero time). Usually the MEM plus 7% FBS used for maintenance during the viral-growth cycle consisted of a 1:1 mixture of fresh MEM plus 7% FBS and conditioned growth medium obtained during the collection of the cells prior to infection.

Infected cells were harvested by centrifugation (10 min at 500 × *g*) after 26 to 30 hr of incubation at 37 C. At this time virus yields were maximal, and the majority of the virus was still cell associated. The cell pellet was washed once with phosphate-buffered saline (PBS) (6), repelleted by centrifugation, and was then stored at −50 C until needed for purification of virus.

Virus purification. Infected cells, either freshly collected or thawed from storage at −50 C, were suspended in PBS at approximately 1 to 2 × 10^8 cells/ml. All operations were performed in an ice bucket. One volume of Genesolv D (Allied Chemical Canada Ltd.) was added, and the cells were homogenized in a Lourdes Multi-Mix homogenizer (Lourdes Instrument Corp., Old Bethpage, N. Y.) fitted with a 50-ml stainless-steel container and blade assembly. Homogenization was carried out for five 1-min periods alternated with 1-min cooling periods. After centrifugation of the homogenate (10,000 × *g*, 10 min, 4 C), the aqueous phase was removed, an equal volume of fresh PBS was added, and the extraction procedure was carried out once more. This cycle was repeated until the aqueous phase was no longer sufficiently turbid to warrant further repetition (ca. 4 to 6 cycles). Usually the aqueous phases from the first two cycles of extraction, which were very turbid, were reextracted with fresh Genesolv D. Virus in the pooled aqueous phase was pelleted by centrifugation (48,300 × *g*, 75 min, 4 C) by using a JA-20 rotor in a Beckman J-21 preparative centrifuge. Pellet material was resuspended in PBS, and CsCl (K & K Laboratories Inc., Plainsview, N. Y.) was added to achieve a solution density of 1.36 to 1.37 g/cc. This was then centrifuged for ~20 hr at 45,000 rpm in a SW50.1 rotor at 5 C in a Beckman L2-65B ultracentrifuge. Three distinct virus-associated bands (top component, 1.29 g/cc; virus, 1.36 to 1.37 g/cc; and clumps, ~1.32 to 1.34 g/cc) were always present in the gradients. These were collected separately from the top, and CsCl was removed by dialysis in the cold against 500 volumes of Tris-chloride buffer (0.1 M, *p*H 8.3) containing 0.1 M NaCl. After dialysis the samples were sonicated (80 kc, 2 min; Sonatron 80, Ultrasonic Devices, Division of Kenyon Electronics, Inc., Jersey City, N. J.) and further purified by velocity sedimentation through 10 to 50% sucrose gradients (in 0.1 M Tris-chloride, *p*H 8.3, buffer containing 1 mM ethylenediaminetetraacetic acid [EDTA]) in a SW25.1 rotor spun at 18,000 rpm for 105 min at 15 C. A single dense band of material was present in each of the tubes, along with minor amounts of impurities. The bands were collected from the top and, after dialysis against 0.1 M Tris-chloride, *p*H 8.3, plus 0.1 M NaCl (500 volumes; 24 hr; 4 C) to remove sucrose, the purified virus was used in the experiments. NaCl was included in the storage buffer to prevent aggregation and loss of activity.

Virus titration. Virus stocks were titrated by plaque formation on monolayers of L cells grown on 60-mm plastic petri dishes (Falcon Plastics, Division of Becton, Dickinson & Co.). Confluent monolayers were drained, 0.2-ml samples of virus diluted in MEM were added to each culture, and virus was allowed to adsorb for approximately 1 hr. During the adsorption period the monolayers were kept in a 37 C incubator gassed with 5% CO_2. The monolayers were then overlaid with 5 ml per plate of overlay agar consisting of 1% agar (Difco Laboratories, Detroit, Mich.), normal strength MEM, 2% FBS, 50 μg of diethylaminoethyl-dextran (Pharmacia, Uppsala, Sweden) per ml, and 30 μg of Mycostatin suspension (GIBCO, Grand Island, N. Y.) per ml. Plates were incubated for 6 or 7 days at 37 C in a 5% CO_2 gas flow incubator. Plaques were stained by overlaying the plates with a 2-ml second layer of 1% agar containing 0.01% neutral red stain (George T. Gurr Ltd., London, England) and incubation at 37 C for an additional 4 to 6 hr.

CHT digestion of virions. Alpha CHT (EC 3.4.4.5; Worthington Biochemical Corp., Freehold, N.J.) was dissolved in buffer (0.1 M Tris-chloride, *p*H 8.3) at 1 or 10 mg/ml. Digestion of reovirions was at 37 C under conditions as specified.

Temperature shock activation of reovirus transcriptase and nucleoside triphosphate phosphohydrolase activity. Samples of purified reovirus, as required, were dispensed in 0.10-ml volumes into 13 × 100 mm disposable glass culture tubes (Corning Glass Works, Corning, N. Y.). These were plunged into a 65.5 C water bath for 15 sec, unless otherwise specified, and then were quenched in an ice bath. Reaction mix as required was then added directly to each activated sample, and the incubation was carried out in the same tube.

RNA polymerase assay. A slight modification of previously reported (4) conditions was used. Each

reaction mix contained in a final volume of 0.250 ml: Tris-chloride, pH 8.3, 20 to 25 μmoles; activated virus equivalent to 3 to 8 μg of protein; adenosine triphosphate, 2 μmoles; guanosine triphosphate, 0.2 μmoles; cytidine triphosphate, 0.2 μmoles; uridine triphosphate, 0.05 μmoles (all from Miles Laboratories, Inc., Kankakee, Ill.); uridine triphosphate-5-^3H, 2.0 μCi, 17.4 Ci/mmole (Schwarz/Mann, Division of Becton, Dickinson & Co., Orangeburg, N. Y.); $MgCl_2$, 2.45 μmole; and other salts as specified. Reaction mixtures were incubated at 37 C for a specified length of time. A 2-ml amount of 5% trichloroacetic acid containing 0.02 M sodium pyrophosphate along with two drops of a 1% (w/v) aqueous solution of bovine serum albumin was added to terminate the reaction and precipitate the polymerized product. Precipitate was collected on glass fiber filters (Whatman GF/A, W&R Balston Ltd., Maidstone, England) by vacuum filtration, washed with cold 5% trichloroacetic acid plus 0.02 M sodium pyrophosphate to remove soluble radioactivity, dried in an oven (10 min at ~250 F), and transferred to linear polyethylene scintillation vials (NEN Canada Ltd., Dorval, P.Q.). The precipitate on the filters was then digested with NCS (Nuclear Chicago Corp., Don Mills, Ontario), and radioactivity was determined by the method of Birnboim (2). Scintillation fluid consisted of Permafluor (Packard Instrument Co., Downers Grove, Ill.) diluted 25× in reagent-grade toluene (Anachemia Chemicals Ltd., Montreal, P.Q.) containing 4.6 ml of glacial acetic acid per gallon. Under these conditions counting efficiencies for tritium ranged between 45 and 55% in a Nuclear-Chicago Mark II or Nuclear-Chicago ISOCAP 300 liquid scintillation spectrometer. Efficiencies were determined by using the sample channels ratio or the external standard channels ratio method.

Nucleoside triphosphate phosphohydrolase assay. The procedure utilizing labeled adenosine triphosphate (adenosine triphosphate-8-^3H; Schwarz/Mann, Division of Becton Dickinson & Co., Orangeburg, N. Y.) as substrate has been described (4).

Spectrophotometric assay of CHT activity. *N*-acetyl-L-tyrosine ethyl ester (ATEE) (Sigma Chemical Co., St. Louis, Mo.) was used as a synthetic substrate to check activity of CHT on a substrate other than reovirus. The procedure used was that described by Schwert and Takenaka (13). Optical density change at 237 nm was monitored in a Cary model 15 recording spectrophotometer (Cary Instruments, Applied Physics Corp., Monrovia, Calif.) with a thermally jacketed sample chamber maintained at 37 C.

Chemicals. Reagent grade $MgCl_2$, CH_3COONa, CH_3COONH_4, and $(NH_4)_2SO_4$ were purchased from J. T. Baker Chemical Co., Phillipsburg, N.J. KCl, NaCl, and NH_4Cl were reagent grade and were purchased from Anachemia Chemicals Ltd., Montreal, P.Q. LiCl was reagent grade and was purchased from the McArthur Chemical Co., Montreal. RbCl was reagent grade and was purchased from Alfa Inorganics, Inc., Beverly, Mass. and given to us by G. Strathdee. Tris-chloride and EDTA-disodium were reagent grade and were purchased from Fisher Scientific Co., Fairlawn, N.J.

RESULTS

Requirement for specific monovalent cations for reovirus transcriptase activity after incubation of purified virions with CHT in vitro. Reovirion core polymerase can be activated in vitro by heat-shocking the virions (3) or by digestion of the outer layer of capsomers by CHT or other proteolytic enzymes (7, 15, 19). During in vitro studies on the properties of the reovirus core transcriptase we routinely activated our virus preparations by the heat shock procedure. For comparison purposes we attempted to activate reovirus, purified as described in Materials and Methods and suspended in 0.1 M Tris-chloride, pH 8.3, containing 0.1 M NaCl, by CHT digestion but failed consistently, even though heat shock activation of the same virus preparations worked with complete reliability. Control experiments established that the CHT preparations used were active. Other control experiments positively confirmed the identity of our virus stocks as being reovirus. Further extensive experiments then led to the discovery that inclusion of KCl in the virus-CHT activation mix facilitated appearance of polymerase activity. This prompted an investigation of the ionic requirements for CHT activation of reovirus transcriptase activity.

To determine whether other salts could substitute for KCl in eliciting reovirus transcriptase activity by CHT, a series of compounds was screened for this ability. Virus was incubated with CHT in the presence of the specified compound and was then assayed for RNA polymerase activity. Each activation mix contained 5 μg of CHT, virus equivalent to 5 μg of viral protein, 1.5 μmoles of NaCl (introduced with 15 μliters of purified virus in 0.1 M Tris-chloride, pH 8.3, plus 0.1 M NaCl), and 15 μmoles (unless otherwise specified) of the compound being tested in a final volume of 0.10 ml of 0.10 M Tris-chloride buffer, pH 8.3. After incubation at 37 C for 30 min, polymerase reaction mix was added, and polymerization was allowed to proceed for 60 min at 37 C. Polymerase activity of each sample was determined as described in Materials and Methods. Results are shown in histogram form in Fig. 1. From the data it is evident that activity occurs after incubation of virus and CHT in the presence of K^+, Rb^+, or Cs^+ ions. Variations in the type of anion

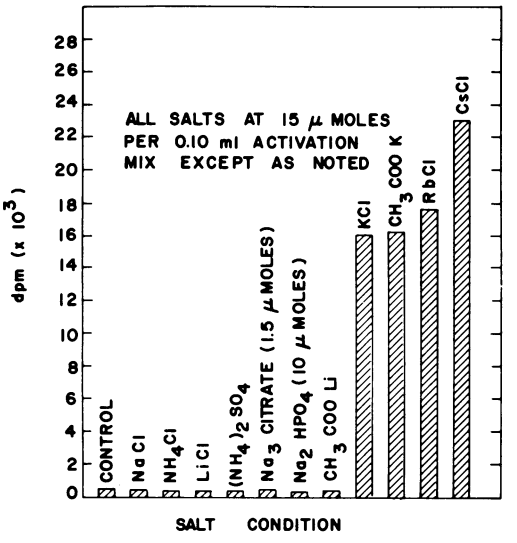

FIG. 1. *RNA polymerase activity after incubation of purified virus, chymotrypsin, and specified salt at 37 C for 30 min in a buffered activation mix as described in the text. After the activation incubation, polymerase reaction substrates were added, and polymerization was carried out for 60 min at 37 C. Trichloroacetic acid-insoluble radioactivity was determined as described in Materials and Methods. The plotted values represent the mean of duplicate determinations.*

do not appear to correlate with changes in activity, indicating that the observed effect on activity arises from properties of the cations present.

To further characterize this requirement of specific cations for transcriptase activity of virions digested by CHT in vitro, activity was studied as a function of concentration of these various cations. Samples of purified reovirus were incubated for 30 min at 37 C with CHT in the presence of a graded series of concentrations of specified salts, and polymerase activity was determined as described above. Figure 2 shows the results. It can be seen that KCl, CsCl, and RbCl are individually capable of eliciting polymerase activity, whereas NaCl and LiCl do not possess this capability. Furthermore, control experiments demonstrated that the observed activity was absolutely dependent on the presence of CHT, indicating that the effect of Cs^+, Rb^+, or K^+ ions was not due to introduction of some nonenzymatic activation process. In other experiments reovirions were digested for extended periods (up to 3 hr at 37 C) with high concentrations of CHT (up to 750 μg/ml) in the presence of Na^+ or Li^+ ions and were found to be inactive in RNA synthesis in vitro. This indicates that Na^+ and Li^+ ions exert an effect on polymerase activity of reovirus digested by CHT which is qualitatively different from that produced by Cs^+, K^+, or Rb^+ ions.

Localization of effect to the activation step. Samples of reovirus were digested with CHT as above, in the presence and absence of KCl (15 μmoles in a 0.100-ml activation volume). After the activation step, polymerase reaction substrates were added, and KCl was added to those samples which had been activated without KCl so that all samples had identical KCl content during polymerization (15 μmoles in a 0.250-ml reaction volume). Identical variations were also carried out with NaCl. Polymerization was allowed to proceed for 60 min at 37 C. Figure 3 shows the results. It can be seen that the addition of KCl after the digestion step resulted in no activity, whereas KCl present during the digestion step permitted expression of the polymerase activity latent within the virion. This result indicates that K^+ ions exert their effect through facilitation of activation by CHT, as opposed to an effect on polymerization per se. It also indicates that no activation could take place under the conditions present during the polymerization incubation, because the CHT was present throughout. This latter observation can be explained in part by the lower concentration of K^+ ions during polymerization and in part by other factors which will become clear in a later section of this paper. Again, no activity was elicited when KCl was omitted and substituted by an equivalent molar amount of NaCl.

Further evidence that the observed cation

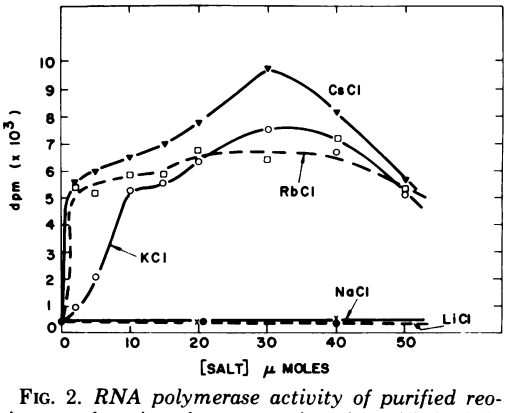

FIG. 2. *RNA polymerase activity of purified reovirus as a function of concentration of specified salt in the activation mixture. The procedure was the same as described in the legend for Fig. 1.*

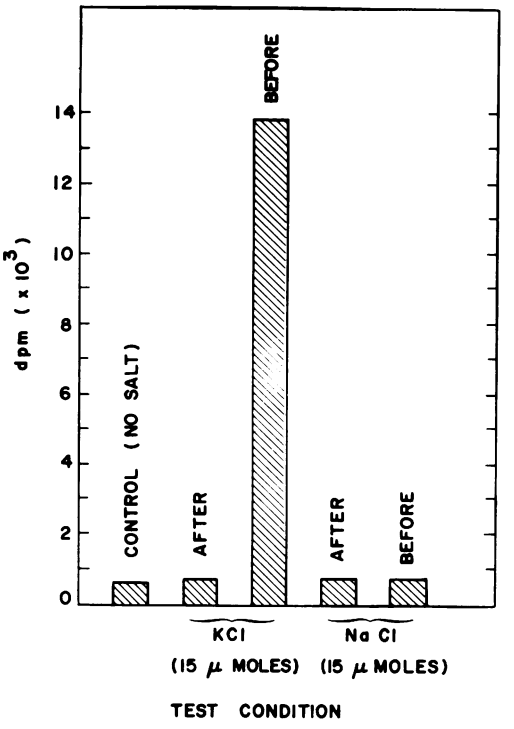

FIG. 3. *Demonstration that effect of KCl is on the activation step. Activation mixes were as described in the text and consisted of samples of purified reovirus, chymotrypsin, and buffer and were incubated for 30 min at 37 C either in the presence or absence of the indicated salt. After the activation incubation, equivalent salt was added to those mixes which had been incubated without the indicated salt, polymerase reaction substrates were added to all, and RNA polymerase activity was determined as described in methods. Controls which did not receive any salt for either activation or polymerization were included. Plotted values are the mean of duplicate determinations.*

effect is on the CHT activation step comes from the fact that heat-shocked virions show no such unique cation requirements for polymerase activity. If the cations exerted their effect on polymerization per se, then one would expect that the requirements would be the same irrespective of the means used to activate the polymerase activity.

Double substrate experiment. It was possible that the observed effect was mediated by influencing of CHT activity per se. To test this possibility, an experiment was performed in which a synthetic substrate, ATEE, was included in an otherwise normal activation mix containing specified monovalent cations, so that CHT activity could be monitored independently of the appearance of RNA polymerase activity. ATEE hydrolysis was determined as described in Materials and Methods, and polymerase activity after incubation for activation was determined as described above. Results are shown in Fig. 4. Curve A shows that in the presence of only Na^+ ion (3.0 μmoles per 0.100 ml) no polymerase activity was evident at 45 min whereas ATEE hydrolysis (curve a) proceeded from zero time and was essentially complete within 5 min. Addition of KCl (15 μmoles per 0.100 ml) at 45 min, followed by further incubation at 37 C, resulted in the appearance of polymerase activity (curve A). Curves B and b show the case where KCl was added at zero time. Polymerase activity appeared after a lag of about 10 min (curve B), and ATEE hydrol-

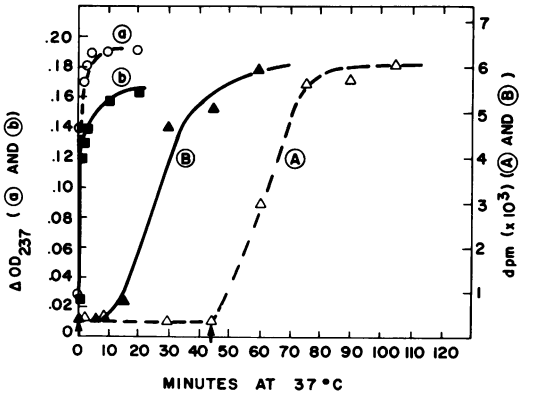

FIG. 4. *Double substrate experiment. Purified reovirus (75 μg/ml), N-acetyl-L-tyrosine ethyl ester (0.0005 M), and specified salt, in 0.1 M Tris-chloride, pH 8.3, were equilibrated at 37 C in the sample chamber of a Cary model 15 recording spectrophotometer. The blank in the reference beam was identical, except that no purified virus was included. At zero time chymotrypsin was added to the sample cuvette to a concentration of 25 μg/ml. Hydrolysis of N-acetyl-L-tyrosine ethyl ester as a function of incubation time was then followed by absorbance change (decrease) at 237 nm; activation of RNA polymerase activity was followed by removing 50-μliter samples from the cuvette, adding polymerase reaction substrates, and proceeding as described in Materials and Methods. Duplicate samples were removed at each sample point. In the first case tested, (a) and (A), only NaCl (equivalent to 3.0 μmoles per 0.10 ml of activation mix) was present for the first 45 min of incubation. At 45 min, KCl was added to the sample cuvette (equivalent to 15 μmoles per 0.10 ml of activation mix). Curve (a) shows the change in optical density at 237 nm; curve (A) shows polymerase activity. In the second case, (b) and (B), KCl (equivalent to 15 μmoles per 0.10 ml of activation mix) was present from zero time. Curve (b) shows the change in optical density at 237 nm; curve (B) shows polymerase activity.*

ysis proceeded from zero time (curve b, essentially identically as in the absence of KCl, curve a). These results clearly demonstrate that CHT was active both in the presence and absence of KCl, but that reovirus RNA polymerase could be activated only in the presence of KCl. This indicates that the observed monovalent cation effect is an effect on the susceptibility of the latent reovirus RNA polymerase to be activated by CHT. Furthermore, there is a lag in the appearance of polymerase activity in the case where KCl is present from zero time during the activation digestion which disappears when activation is facilitated by the addition of KCl after a preincubation period in the absence of KCl (compare curves B and A). This observation suggests that during the preincubation in the absence of KCl some digestion of the outer coat of reovirus is occurring, but that digestion is blocked at some point prior to activation of the polymerase.

Antagonism of K^+ or Cs^+ effect by Na^+ or Li^+ ions. Since reovirus transcriptase could not be activated by CHT digestion in the presence of Na^+ or Li^+ ions alone, it was of interest to determine if this was due to lack of a positive effect of these ions or if they exerted a negative effect resulting in inhibition of activation. This question was approached by performing activation experiments in the presence of combinations of ions. A series of activation mixes was set up in which a fixed concentration of facilitating ion (K^+ or Cs^+) was combined with a graded series of concentrations of nonfacilitating ion (Na^+ or Li^+). The nonfacilitating ion was added before activation in one series and after activation in a parallel series to distinguish between possible effects on activation and polymerization per se. Results are shown in Fig. 5. Data in panel A are for the case where the facilitating ion was K^+ (15 μmoles in 0.100 ml). It can be seen that Na^+ or Li^+ added before activation very effectively suppressed the K^+ facilitation of activation, with Li^+ being more effective on a molar basis than Na^+. Addition of these ions after activation in the presence of K^+ ion resulted in marked stimulation of polymerization by Li^+ and essentially no effect by Na^+. These data clearly demonstrate a separation of the effects of monovalent cations on activation of polymerase activity by CHT from their effects on polymerization per se. Results of a similar experiment in which Cs^+ was the facilitating ion (15 μmoles in 0.100 ml) are shown in panel B. It is evident by comparing the two panels that Na^+ or Li^+ were required at much higher concentrations to suppress Cs^+ facilitation of activation by CHT

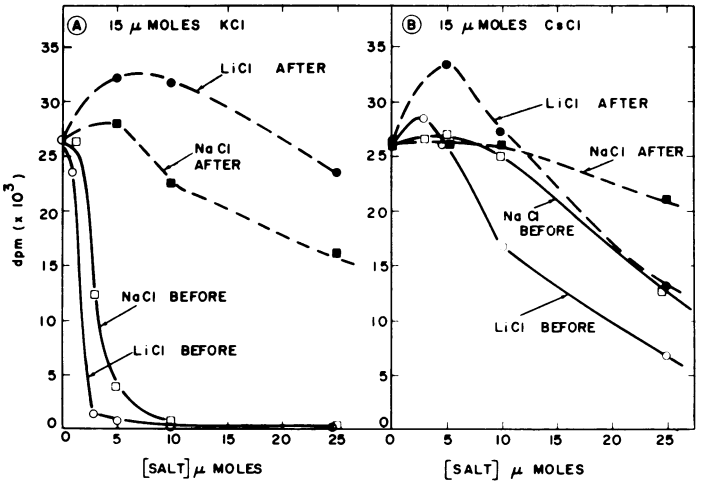

FIG. 5. *Antagonistic effect of Na^+ or Li^+ on K^+ or Cs^+ facilitation of polymerase activation by chymotrypsin (CHT) digestion. Activation mixes were set up containing purified virus (5 μg), CHT (10 μg), KCl (15 μmoles), and (in the "before" tubes only) the indicated amount of the specified nonfacilitating salt in 0.10 ml of 0.10 M Tris-chloride, pH 8.3. Digestion was carried out at 37 C for 30 min, after which the indicated amount of the specified nonfacilitating salt was added to the "after" tubes. Polymerase reaction substrates were then added to all the tubes, and incubation at 37 C was continued for an additional 60 min. Trichloroacetic acid-precipitable radioactivity was then determined as described in Materials and Methods. All points were done in duplicate.*

than were required to suppress K^+ facilitated activation by CHT. Addition of Na^+ or Li^+ after activation yielded results similar to those obtained in the case where K^+ was the facilitating ion. The above results clearly demonstrate an antagonistic interaction between the facilitating and nonfacilitating ions with regard to their effect on the activation of reovirus RNA polymerase activity by CHT.

The Na^+ antagonism of the K^+ facilitation of activation was investigated in more detail. Activation mixes containing CHT, purified virus, and various amounts of NaCl and KCl were set up as described above. After digestion for 30 min at 37 C, polymerase reaction substrates were added, and activity was determined. In Fig. 6, activity versus concentration of KCl for different levels of NaCl is shown. This demonstrates that the level of NaCl present markedly influences the K^+ facilitation of activation.

Several other compounds were tested for an effect on the facilitation of polymerase activation by CHT in the presence of K^+, Rb^+, or Cs^+. Table 1 shows the data. It should be pointed out that this experiment was carried out with virus suspended in 0.1 M Tris-chloride, *p*H 8.3, containing 0.1 M KCl instead of the usual 0.1 M NaCl. This appears to alter the quantitative relationships between pairs of facilitating and nonfacilitating ions somewhat, but the qualitative results are unchanged. From the data it is evident that K^+ facilitated activation is readily inhibited by the nonfacilitating monovalent cations (Na^+, Li^+, NH_4^+), divalent cations (Mg^{2+}, Ca^{2+}), nucleoside triphosphates (adenosine triphosphate, guanosine triphosphate, and cytidine triphosphate at 0.6, 0.2, and 0.2 μmoles per 0.10 ml of activation mix, respectively) and to a slight extent, sucrose (10%). These data explain the earlier observation (Fig. 3) that K^+ added before the activation digestion facilitated activation, whereas the same amount of K^+ added after the activation digestion, at which time polymerase substrates (nucleoside triphosphates and Mg^{2+}) were also added, failed to facilitate activation. With Rb^+ or Cs^+ as the facilitating ions there was no inhibition by the nonfacilitating monovalent cations at the concentrations employed. In fact there appeared to be a stimulation of activity, but this was due to an effect on polymerization per se since the nonfacilitating ions by themselves were not capable of facilitating the appearance of activity (last column). Mg^{2+} substantially inhibited activation in the presence of Rb^+, although less than with K^+, but not at all in the case of Cs^+. Ca^{2+}, on the other hand, very effectively inhibited activation with all of the facilitating ions. Similarly, with nucleoside triphosphates and sucrose there was a greater

TABLE 1. *Effect on the facilitation of polymerase activation by chymotrypsin in the presence of cations*

Test substance	Activation mix (μmoles)	Facilitating cation[a] (15 μmoles)			
		Cs^+	Rb^+	K^+	None
NaCl	5	6,340[b]	6,047	1,768	199
	15	6,198	—	—	—
LiCl	5	7,277	5,599	133	199
	15	5,206	—	—	—
NH_4Cl	5	7,154	5,990	2,736	270
	15	6,591	—	—	—
$MgCl_2$	0.5	6,422	1,716	118	175
$CaCl_2$	0.5	190	135	66	69
[c]NTP	1.0	5,778	4,644	2,974	247
[c]NTP plus $MgCl_2$	1.0	5,202	3,554	176	140
	1.0				
Sucrose	(10%)	5,061	3,851	4,419	1,042
None	—	5,684	4,710	4,857	323
KCl	15	—	—	—	4,773

[a] Chloride salts were used.

[b] Disintegrations per minute incorporated into trichoroacetic acid-precipitable material in 60 min incubation at 37 C. Mean of duplicate determination.

[c] Nucleoside triphosphate phosphohydrolases (NTP) were comprised of adenosine triphosphate (0.6 μmole), guanosine triphosphate (0.2 μmole), and cytidine triphosphate (0.2 μmole).

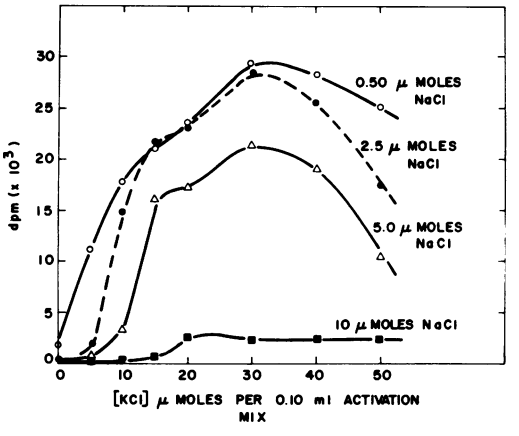

FIG. 6. *Polymerase activity as a function of concentration of KCl, present with a specified constant amount of NaCl, in the activation mix. Each activation mix contained in a final volume of 0.10 ml of 0.1 M Tris-chloride, pH 8.3: purified virus (5 μg), chymotrypsin (10 μg), and the indicated amounts of KCl and NaCl. Digestion was for 30 min at 37 C, after which polymerase reaction substrates were added, and polymerase activity was determined as described (60 min at 37 C incubation, all points in duplicate).*

degree of inhibition of Rb^+ facilitation than of Cs^+ facilitation. From data such as these it is possible to order the nonfacilitating monovalent cations with respect to their potential to antagonize the effect of a given concentration of a particular facilitating monovalent cation in the sequence $NH_4^+ < Na^+ < Li^+$. Similarly, the facilitating monovalent cations can be ordered with respect to their potential to facilitate activation in the presence of a constant amount of inhibitory monovalent cation in the sequence $K^+ < Rb^+ < Cs^+$. An important feature of these data is the efficacy of Cs^+ in facilitation of activation in the presence of the various interfering substances. Because isopycnic centrifugation on CsCl gradients is frequently the last step in purification of reovirus for use in the study of the viral transcriptase, the efficacy of Cs^+ in facilitating activation means that, unless adequate precautions are taken, sufficient Cs^+ may be present in the purified virus preparation to facilitate activation and thereby mask the monovalent cation requirement. This may be the reason why the requirement for monovalent cations described here has not previously been detected in those laboratories which regularly use CHT digestion as a means of activating reovirus transcriptase, although other factors may be present as well.

Effect of varying CHT concentration. The relative amounts of virus and CHT could be an important factor in determining the activation process. Therefore, an experiment was carried out to measure activation as a function of CHT concentration for a fixed amount of virus. A series of duplicate activation mixtures was set up, each containing 3 μg of viral protein, 1.0 μmole of NaCl (introduced with the 10 μliters of purified virus suspension), 15 μmoles of either KCl or CsCl as specified, and an indicated amount of CHT in a total volume of 0.10 ml of 0.10 M Tris-chloride, *p*H 8.3. The tubes were incubated for 30 min at 37 C to activate the virus, after which polymerase reaction substrates were added, and polymerization was carried out for 60 min at 37 C. Figure 7 shows the resulting data. In the case where Cs^+ was the facilitating cation, activity reached a plateau at approximately 10 μg of CHT per activation mix and stayed at the maximum as the CHT level was increased up to 75 μg of CHT per activation mix. This saturation type result agrees with that reported by Skehel and Joklik (19). On the other hand, when K^+ served as the facilitating cation, the activity versus

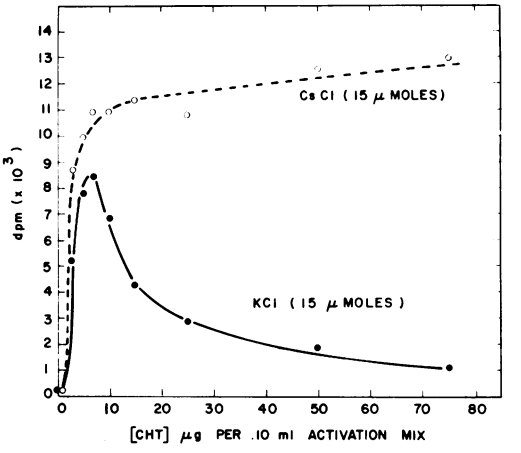

FIG. 7. *Effect of varying chymotrypsin (CHT) concentration. Activation was carried out with variable amounts of CHT as described in the text, after which polymerase activity was determined.*

concentration of CHT curve passed through a maximum at ~ 8 μg of CHT per activation mix and then decreased with increasing CHT concentration as shown. The decrease observed with increasing amounts of CHT was not the result of destruction of the active cores by the high levels of CHT present, but represented a failure to activate because heat shock after the digestion step elicited the full level of activity (data not shown). In other experiments (not shown), it was found that the optimal concentration of CHT for a fixed amount of virus was determined by the level of KCl present in the activation mix, with increased levels of KCl moving the optimum to higher levels of CHT. The basis for this K^+-CHT interaction is not clear to us, and further experiments along these lines are in progress. Dialysis of the CHT against salt-free 0.1 M Tris-chloride, *p*H 8.3, to remove any salts possibly carried over from the manufacturing procedure, did not alter the results.

With Rb^+ as the facilitating cation, the activity versus CHT concentration curve (data not shown) was found to be the same type as that observed with Cs^+.

Effects of various salts on polymerization per se. During the course of these studies the various test salts were also examined for possible effects on polymerization per se by adding them to the reaction mix *after* the activation incubation in the presence of facilitating ion had been completed. The conclusions to be drawn from the bulk of our data (not included here) are that (i) certain monovalent

cations such as Li^+, K^+, and NH_4^+ stimulate polymerization per se to various degrees, and (ii) that the species of anion present markedly influences the polymerization. In particular Cl^-, $HPO_4^=$, and $SO_4^=$ ions markedly inhibit the reaction. Acetate anions, on the other hand, appear to be noninhibitory. Figure 8 demonstrates these points. This indicates the desirability of using acetate as the anion form of any salts which are to be added to the polymerase reaction mixes in concentrations where Cl^-, $SO_4^=$, or phosphate anions may be inhibitory.

Activation of nucleoside triphosphate phosphohydrolase activity by CHT has same requirement for specific monovalent cations. Reovirions are known to possess a nucleoside triphosphate phosphohydrolase activity which is latent in the intact virion but can be activated by heat shock or CHT digestion of the virions under appropriate conditions (4, 9). Activation requirements for nucleoside triphosphate phosphohydrolase activity by CHT digestion were checked to see whether any differential effects for activation of the nucleoside triphosphate phosphohydrolase and polymerase activity could be demonstrated. Accordingly, samples of purified reovirus were digested with CHT in the presence of indicated amounts of specified salts, and each sample was then assayed for both nucleoside triphosphate phosphohydrolase and RNA polymerase activity in vitro. Fig. 9 shows that activation of nucleoside triphosphate phosphohydrolase activity by CHT digestion of reovirions has the same requirement for specific monovalent cations as does RNA polymerase activity.

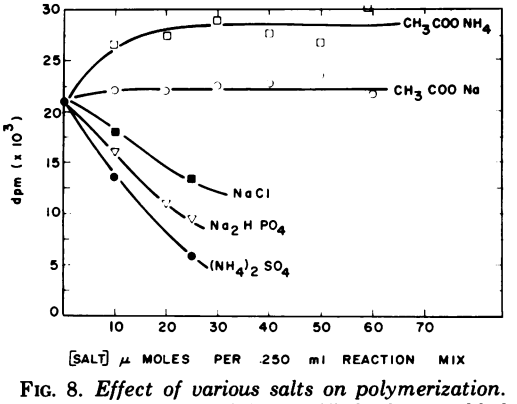

FIG. 8. *Effect of various salts on polymerization. The indicated amount of the specified salt was added along with polymerase reaction substrates after the virus (5 μg per activation mix) had been digested for 30 min at 37 C by chymotrypsin (10 μg per activation mix) in the presence of KCl (15 μmoles per 0.100 ml of activation mix). The polymerization incubation was for 60 min at 37 C. All points are in duplicate.*

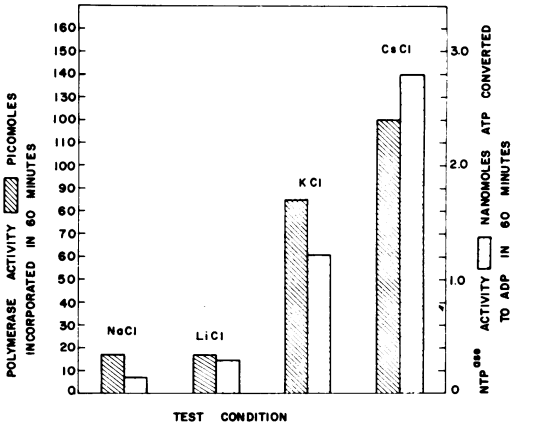

FIG. 9. *Comparison of activation requirements of nucleoside triphosphate phosphohydrolase and RNA polymerase activities in purified reovirions. Samples of purified virus (~15 μg) were digested at 37 C for 30 min by chymotrypsin (~5 μg) in the presence of KCl (10 μmoles), NaCl (10 μmoles), LiCl (10 μmoles), or CsCl (5 μmoles) in a 0.10-ml volume of 0.10 M Tris-chloride, pH 8.3. Each activated sample was then divided into two equal portions. One portion was assayed for RNA polymerase activity and the other portion was assayed for nucleoside triphosphate phosphohydrolase activity as described in methods. All points are in duplicate.*

DISCUSSION

The data presented above demonstrate that the activation of the RNA polymerase and nucleoside triphosphate phosphohydrolase activities, present in a latent form in intact reovirions, by digestion of the virions with CHT in vitro has an absolute requirement for specific monovalent cations, namely K^+, Rb^+, or Cs^+. Na^+, Li^+, or NH_4^+ ions exert an antagonistic effect towards this facilitation of activation by the former group of ions. This suggests that these two groups of monovalent cations may be in competition for a common site, or sites, which determines whether the digestion with CHT can proceed to the point required for activation of the latent enzymes within the virion. The effect of monovalent cations appears to be mediated via some mechanism which controls the susceptibility of at least some portion of the virion to digestion by CHT, since the double substrate experiment shows that CHT activity per se is essentially unaffected by the variations in monovalent cation required to control the ac-

tivation. It appears reasonable to speculate that specific monovalent cations influence conformations of at least some viral polypeptides in such a fashion that the Na^+-, Li^+-, or NH_4^+-induced conformation(s) contains certain bonds in a CHT-resistant state, whereas in the K^+-, Rb^+-, or Cs^+-induced conformation these bonds are susceptible to digestion. Such polypeptide(s) would probably serve, in the intact virion, as part of the mechanism for suppression of the polymerase and nucleoside triphosphate phosphohydrolase activities.

Kinetics of K^+ facilitated activation of polymerase activity differ depending on whether or not the virions have been preincubated with CHT in the absence of facilitating ion. It appears that the preincubation removes the lag period which is present with no preincubation. This strongly suggests that a limited amount of digestion by CHT can take place in the absence of facilitating ion. In separate studies, which are still in progress, we have confirmed this, and the results of that series of experiments will be published in a separate communication.

Extrapolation of the observed monovalent cation effect in vitro to the situation involving viral infection of cells reveals some extremely interesting and important implications. Under physiological conditions there is normally a high K^+ ion concentration intracellularly and a high Na^+ concentration extracellularly. This would mean, if our extrapolation is valid, that reovirions cannot be uncoated to the level of polymerase activity by enzymatic digestion until they are in an intracellular environment. In effect this property of the virion enables it to sense whether it is in an intracellular or an extracellular environment. Preliminary experiments in our laboratory have indicated that after CHT digestion of intact reovirions in the presence of nonfacilitating monovalent cations (Na^+ or Li^+) the infectivity of the virions is fully retained and may in fact be considerably enhanced (*manuscript in preparation*). Because reovirions which have been digested down to the level of active cores in vitro lose essentially all their potential for infecting cells (7, 20; also our own unpublished data), the above mechanism for preventing premature uncoating would have obvious utility in increasing the probability of successful passage of the virus particle to a new host cell.

An additional aspect of this phenomenon is that it should be experimentally useful in the study of the activation process by CHT digestion in vitro. Such studies are currently in progress.

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