Specific Alterations of Coxsackievirus B3 Eluted from HeLa Cells¹

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After the attachment of radioactive coxsackievirus B3 to HeLa cells at 0 C and subsequent incubation at 37 C, 50 to 80% of attached virus radioactivity was eluted from the cells within 1 hr. Eluted virus had a buoyant density of 1.21 in a potassium tartrate gradient, sedimented more slowly than native virus in sucrose gradients, was resistant to ribonuclease, was unstable in CsCl centrifugation, and did not re-attach to uninfected cells. Electrophoretic studies of sodium dodecyl sulfate-disrupted B3 virus in sodium dodecyl sulfate-polyacrylamide gels revealed four radioactive virus polypeptides (VP 1 to 4), of which the three largest migrated slightly faster than their poliovirus T1 counterparts. In contrast, electrophoretic analysis of eluted virus, after banding in a tartrate gradient or pelleting by centrifugation, showed the absence of the fastest migrating polypeptide, VP 4. VP 4 was recovered in the supernatant fluid when the eluted virions were removed by high-speed centrifugation. The results indicate that VP 4 is located at the surface of the native virion, and its dissociation from the capsid may represent the first specific alteration of the virion after virus-receptor interaction at the cell surface.

The phenomenon of elution or sloughing of radioactive poliovirus from the surface of host cells has received little attention since it was first described (10, 11). Recently, we extended these studies and found that from 50 to 80% of cell-associated labeled coxsackievirus B3 also elutes from HeLa cells at 37 C (6). This latter finding was unexpected, because poliovirus and members of coxsackievirus group B attach to separate receptors on the surface of HeLa cells (5, 7). The present report shows that the characteristics of eluted coxsackievirus B3 resemble those of eluted poliovirus. In addition, evidence is given that the low-molecular-weight viral protein, designated VP 4, is dissociated from the eluted virion without a concurrent release of viral ribonucleic acid.

MATERIALS AND METHODS

HeLa cells. Cells were propagated in suspension with double strength Eagle's minimal essential medium (MEM; reference 9) supplemented with 5% horse serum, 2% calf serum (CaS-2), and 2 μ g of insulin

per ml (3). HeLa cells for virus plaque assays were cultured as monolayers in plastic petri dishes or rectangular 2-oz (59 ml) bottles as described previously (6).

Viruses and virus purification. The origin of the Nancy strain of coxsackievirus B3 and the Mahoney strain of poliovirus T1 used in this study was described earlier (7). The viruses were grown in suspension cultures of HeLa cells with an input virus multiplicity of 10 to 50 PFU/cell. The cells were centrifuged at $900 \times g$ for 15 min, the supernatant medium was discarded, and the cells were suspended in CaS-2-MEM with 0.02 M *N*-2-hydroxyethylpiperazine-*N'*-2'-ethanesulfonic acid (HEPES) buffer (pH 7.5), 9 mM NaHCO₃, 2 μ g of insulin per ml, penicillin (100 units/ml), and streptomycin (100 μ g/ml) to a final concentration of 2×10^7 cells/ml. Virus was added, the cells were incubated for 1 hr at 37 C on a magnetic stirrer, and medium with or without radioactive isotope was added to give a final concentration of 2×10^6 cells/ml. Viruses labeled with ³²P-phosphate, ³H-amino acids, or ¹⁴C-valine were grown in MEM containing citrate in place of phosphate, MEM with 0.1 amino acid concentration, and MEM devoid of valine, respectively. The infected cells were incubated for an additional 8 hr in suspension at 37 C, harvested by centrifugation, washed once in phosphate-buffered salt solution devoid of magnesium and calcium (PBSA; reference 8), resedimented, and re-suspended in PBSA at a concentration of approximately 5×10^7 cells/ml. Less than 1% of the virus yield was found in the extracellular fluids. Intracellular

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virus was released by three cycles of freezing and thawing, and the cell debris was removed by centrifugation at $900 \times g$ for 15 min. Virus was purified by the method of Öberg and Philipson (17). Samples (3.0 ml) of supernatant fluid were overlaid on 1.8-ml cushions of CsCl (1.40 g/ml) and centrifuged for 3 hr at 38,000 rev/min in a swinging bucket rotor (SW 39) in an ultracentrifuge. The virus-rich band was collected in a 10-drop fraction after puncture of the bottom of each tube. These fractions were pooled, dialyzed against PBSA, and centrifuged at $900 \times g$ for 15 min to remove coarse particulates. The virus-containing supernatant fluid was added to 2.07 g of solid cesium chloride, brought to a final volume of 4.5 ml with PBSA, overlaid with mineral oil, and centrifuged for 16 hr at 38,000 rev/min (SW 39 rotor) to equilibrium. The bottom of the tube was punctured, and three-drop fractions were collected into 0.1 or 0.3 ml of PBSA. The two or three fractions found to contain virus by radioactive or immunodiffusion assays were pooled, dialyzed against PBSA, and frozen in small samples in the vapor phase of a liquid-nitrogen refrigerator. The general characteristics of purified virus were similar to those described previously (6, 11). A previously described plaque method for assay of virus infectivity (7) was performed in triplicate or quadruplicate.

Preparation of cell-associated virus and eluted virus. HeLa cells grown in suspension were washed three times in PBSA by alternate centrifugation and resuspension techniques, incubated for 15 min in 0.02% ethylenediaminetetraacetic acid (EDTA), dispersed in PBSA at 37 C, and counted in a hemacytometer. The desired number of cells was deposited by centrifugation and placed on ice. The cells were resuspended in saline buffered with HEPES (0.05 M) to give a final concentration of 4×10^7 cells/ml after the addition of virus. The input multiplicity of labeled virus did not exceed 200 PFU/cell. Virus-cell interaction was allowed for 1 or 2 hr at 0 C, the amount of unattached virus was reduced by three washes with centrifugation in 10-ml amounts of cold buffered saline, and the cells with attached virus were resuspended in cold buffered saline. The amount of cell-associated virus (CAV) was determined from radioactive assays of all wash fluids and cell-associated label. The cell suspension was placed at 37 C for 1 hr for elution of virus radioactivity. No significant difference in the amount of eluted virus (EV) was obtained when the final concentration of cells was adjusted between 5×10^6 and 10^8 cells/ml. A cell concentration in excess of 10^8 cells/ml yielded less EV. The EV in the fluid phase was separated from the cells by centrifugation at $900 \times g$ for 10 min at 4 C and assayed for infectivity and radioactivity.

Assay for radioactivity. Samples containing ^{32}P were counted by Cerenkov radiation in 10 ml of 0.1 M NaOH with the ^3H setting in a liquid scintillation spectrometer (2) or, in double-label experiments, with ^3H by scintillation in a dioxane-cabosil scintillation base. In some experiments, ^3H and ^{14}C samples were solubilized in 0.5 ml of 0.1 M NaOH and 10 ml of a toluene liquid scintillation mixture containing 10 ml of B10-SOLV-BBS3 per 100 ml of solution (Beck-

man Instruments, Inc., Fullerton, Calif.). In experiments with double labels, appropriate replicate samples employing each isotope separately were assayed for correction of spill of counts in the respective channels.

Reagents. ^{14}C -L-valine was purchased from New England Nuclear Corp., Boston, Mass. as 1.8 mCi/mg; carrier-free ^{32}P -phosphate, 35 to 70 mCi per mg of phosphorus, was obtained from Radiochemical Center, Amersham, England; and reconstituted protein hydrolysate of ^3H -amino acids containing 1 mCi/ml in 0.01 M HCl was obtained from Schwarz BioResearch, Inc., Orangeburg, N.Y.

Polyacrylamide gel electrophoresis. The samples in PBSA were diluted twofold in a virus-solubilizing solution, which contained 3% sodium dodecyl sulfate (SDS) in 0.02 M sodium phosphate buffer (pH 7.1), 0.03 M EDTA, and freshly added 0.3 M mercaptoethanol. The mixture was prepared in small conical polypropylene tubes with hinged caps and heated in a boiling-water bath for 2.5 min. The samples were cooled to room temperature, and sucrose was added to a final concentration of 10% before 0.1- to 0.3-ml samples were layered onto the gels. The gels were casted in plastic tubes (20 by 1.0 cm) under a layer of water (14). The gels contained 10% acrylamide and 0.25% bisacrylamide in 0.1% SDS and 0.1 M sodium phosphate buffer (pH 7.2). Each reagent was recrystallized before used (17). The buffer used for electrophoresis contained 0.1 M sodium phosphate (pH 7.1) with 0.1% SDS and 0.005 M EDTA. The gels were prerun for 17 hr at 70 v with approximately 1 ma per cm of gel in a modified Buchler electrophoresis apparatus with the buffer recirculated by a vibrostaltic pump. The samples were usually electrophoresed for 17 hr at 70 v and approximately 1 ma per cm of gel. The gels were cut manually in 2-mm slices in a modified Maizel apparatus (13) by a fine wire, and each slice was crushed in 0.5 ml of 0.1 M NaOH, was left over night, and received 10 ml of toluene scintillation mixture before it was counted.

RESULTS

Attachment and elution of labeled coxsackievirus B3. Two separate purified preparations of coxsackievirus B3, labeled with ^{32}P and a mixture of ^3H amino acids, respectively, were mixed and tested for their ability to attach to HeLa cells at 0 C. HeLa cells were washed, dissociated by EDTA (0.02%), and suspended in saline buffered with HEPES (0.05 M, pH 7.0), to give a final cell concentration of 4×10^7 /ml after addition of virus. The virus inoculum contained 3.5×10^8 PFU (input multiplicity of 18 PFU/cell). At intervals of incubation on ice, samples were removed and diluted 1:10 in PBSA, the cells were sedimented, and the supernatant phase was assayed for infectivity and radioactivity. Figure 1 shows that both radioactive labels coincided and attached at a rate comparable to that found for PFU.

FIG. 1. Correlation between the attachment rates of coxsackievirus B3 radioactivity (CPM) and plaque-forming units (PFU) to HeLa cells in suspension. A mixture of ^{32}P - and ^3H -amino acid-labeled virus preparations was used. Symbols: X, ^{32}P -labeled coxsackievirus B3; O, ^3H -amino acid-labeled coxsackievirus B3.

In another experiment, CAV was prepared as described above with ^{32}P -coxsackievirus B3 and HeLa cells. After removal of unattached virus, duplicate samples of cells were resuspended in buffered saline at a final concentration of 2×10^7 cells/ml. One tube was incubated at 37°C and the other at 0°C. At intervals, 0.1-ml samples were removed and diluted 10-fold in saline, the cells were removed by centrifugation ($700 \times g$), and the supernatant fraction was assayed for infectivity and radioactivity. Figure 2 shows that 60% of the total CAV radioactivity was released into the fluid phase during incubation at 37°C, whereas at 0°C the radioactivity remained cell-associated. No elution of virus infectivity was observed at either temperature when the samples were tested by the routine plaque assay. However, results reported elsewhere (6) showed that the eluted virus preparation contained its full complement of infectious ribonucleic acid (RNA). In addition, when ^{32}P -labeled eluted virus preparations were tested for ribonuclease (200 $\mu\text{g}/\text{ml}$) sensitivity of the label, it was found that 80 to 90% of the label was acid-precipitable after digestion for 30 min at room temperature.

Analysis of eluted coxsackievirus B3 by density gradient centrifugation. In previous studies, Joklik and Darnell (11) found that eluted poliovirus was unstable when centrifuged in cesium chloride gradients. We found a similar instability for eluted coxsackievirus B3. An experiment was done to determine whether eluted coxsackievirus B3 possessed a lighter buoyant density in a potassium tartrate gradient as was found for eluted poliovirus (10). A mixture of coxsackievirus B3 labeled with ^{32}P and ^3H -amino acids was attached and eluted from HeLa cells as described above. The

cells were sedimented by centrifugation ($700 \times g$) for 10 min, and 0.5 ml of the supernatant fluid containing eluted virus was layered over a pre-formed gradient of potassium tartrate (3.5 ml), ranging from 300 to 600 mg/ml. A separate mixture of labeled normal virus was applied to a second tartrate gradient to serve as control. The samples were centrifuged for 4 hr at $80,000 \times g$, and two-drop fractions were collected from the bottom of each tube. Samples of each of the fractions were assayed for ^3H and ^{32}P activity, and the density of selected fractions was determined from measurements of the refractive index.

The results of this experiment (Fig. 3) show that in both the eluted and normal virus preparations, the ^3H -amino acid label and the ^{32}P label coincided. Thus, further evidence was provided that the eluted virus contained RNA. The eluted virus was found to have a buoyant density of 1.21.

FIG. 2. Effect of temperature on the rate of elution of coxsackievirus B3- ^{32}P radioactivity from HeLa cells at pH 7.0.

FIG. 3. Comparative sedimentation of eluted and normal coxsackievirus B3 in potassium tartrate density gradients (300 to 600 mg/ml). A mixture of ^{32}P - and ^3H -amino acid (^3Haa)-labeled virus was used in each preparation.

This was in marked contrast to that of normal virus, which sedimented to the bottom of the tartrate gradient.

In another experiment, eluted coxsackievirus B3 was prepared from ^3H -amino acid-labeled virus and compared with ^{14}C -amino acid-labeled normal B3 virus for sedimentation in preformed sucrose gradients (5 to 25% sucrose with 0.01 M HEPES buffer, pH 7.0, and 0.01 M EDTA). Three sucrose gradients were prepared (3.5 ml each) in cellulose acetate tubes and overlaid with 0.5-ml amounts of eluted virus, normal B3- ^{14}C -amino acids, and a mixture of eluted and normal virus, respectively. The samples were centrifuged for 90 min at 30,000 rev/min, two-drop fractions were collected from the bottom of each gradient, and 0.1-ml samples of each fraction were assayed for radioactivity.

The results (Fig. 4) reveal that normal virus sedimented at a faster rate than eluted virus. There was no difference in the sedimentation rates of the respective virus preparations when they were centrifuged separately (results not shown). The findings that eluted coxsackievirus B3 had a lighter buoyant density in a potassium tartrate gradient and that it sedimented more slowly in sucrose gradients than normal B3 virus are in close agreement with those observed by Fenwick and Cooper (10) for eluted poliovirus.

Inability of eluted coxsackievirus B3 to attach to HeLa cells. Two preparations of eluted B3 virus labeled with ^3H -amino acids and ^{32}P were mixed and tested for their ability to attach to HeLa cells at 0 C. A mixture of ^3H -amino acid-

FIG. 4. Comparative cosedimentation of eluted [^3H -amino acid (^3Haa)] and normal [^{14}C -amino acid (^{14}Caa)] coxsackievirus B3 in a sucrose gradient (5 to 25%).

and ^{32}P -labeled normal virus served as the control. HeLa cells grown in suspension cultures were washed, and replicate samples were suspended in HEPES-buffered saline (pH 7.0) to give a final cell concentration of 6×10^7 cells/ml after addition of virus. At intervals of incubation on ice, 0.1-ml samples were diluted 1:10 in buffered saline, the cells were sedimented by centrifugation, and both phases were assayed for radioactivity. The results of these assays are given in Fig. 5. The eluted virus did not attach to normal HeLa cells, and both the ^3H -amino acid and ^{32}P labels coincided in the respective preparations. Attempts to dissociate the eluted virus from cellular material, which may have remained bound to the eluted virus, by the use of glycine buffer (pH 1.5) at 0 C (18) disrupted the virus. Thus, the inability of eluted virus to attach to the cellular receptors may have resulted from masking of sites on the eluted virus by cellular material.

Loss of VP 4 from coxsackievirus B3 after elution from HeLa cells. To characterize further the eluted virus, both native and eluted coxsackievirus B3 species labeled with radioactive amino acids were disrupted by heating for 2.5 min in a boiling-water bath in SDS, and their polypeptide content was analyzed by SDS-polyacrylamide gel electrophoresis as described above. For standardization of the method, the polypeptide profile of poliovirus T1 first was compared with that of coxsackievirus B3.

To a mixture of 10 μl of B3- ^3H -amino acids and 10 μl of poliovirus T1- ^{14}C -valine was added 30 μl of PBSA and 50 μl of solubilizing solution. The preparation was heated in a boiling-water bath, carefully layered onto a SDS-10% polyacrylamide gel

FIG. 5. Inability of eluted coxsackievirus B3 to attach to HeLa cells. A mixture of ^{32}P - and ^3H -amino acid-labeled virus was used in each preparation, and the results are depicted by single points, because the different radioactivities coincided in the respective preparations.

FIG. 6. Comparative SDS-acrylamide gel electropherogram of a mixture of coxsackievirus B3- ^3H -amino acids (^3Haa) and poliovirus T1- ^{14}C -valine preparations. The origin is to the left, with polypeptide migration toward the anode at the right side of this and subsequent figures.

(16 by 1 cm), and subjected to electrophoresis for 17 hr at 70 v and 1 ma per cm of gel. Equivalent samples of coxsackievirus B3 and poliovirus T1 were assayed separately to serve as a control.

The electrophoretic profile of the mixture of poliovirus T1 and coxsackievirus B3 (Fig. 6) revealed that coxsackievirus B3 possesses four polypeptides similar to those of poliovirus T1 (16). Similar profiles were obtained for each of the viruses when they were subjected to electrophoresis separately. These results permitted a determination of the approximate molecular weights of each of the coxsackievirus B3 polypeptides based on the values reported for those of poliovirus T1 (16). The values for coxsackievirus B3 polypeptides, as derived from a linear plot of the log of the molecular weight of the poliovirus polypeptides against the gel fraction, were approximately VP 1 = 29,000; VP 2 = 25,000; VP 3 = 21,000; and VP 4 = 5,500. A value of 38,000 was also obtained for VP 0, which was usually found as a minor contaminant of the purified virions.

A preparation of eluted coxsackievirus B3 labeled with a ^3H -amino acid mixture was prepared as described above. A sample of the preparation (0.6 ml) was pelleted in a microtube (Spinco no. 305528) at $115,000 \times g$ for 2 hr in a swinging-bucket rotor (SW 39). A preparation of purified, native ^3H -amino acid-labeled virus was diluted in buffered saline (20 μl of virus plus 0.58 ml of saline) to serve as control of the centrifugation procedure. Assay of the distribution of radioactivity between the supernatant phase and the pellet showed approximately 5 to 10% of the counts remaining in the supernatant frac-

tions. The pellets were suspended in 0.2 ml of virus-solubilizing solution and assayed for polypeptide content by polyacrylamide gel electrophoresis as described previously. Coxsackievirus B3 labeled with ^{14}C -valine was added to each sample before heating and coelectrophoresed for 16 hr at 65 v.

The results of the electrophoresis profiles of native and eluted coxsackievirus B3 are given in Fig. 7 and 8, respectively. Figure 7 depicts comparable profiles of virus proteins with the two labels, except that somewhat less label was found in VP 4 labeled with valine. Figure 8 reveals that eluted virus had lost the low-molecular-weight protein designated VP 4. The loss of VP 4 from the coxsackievirus virion after elution from HeLa cells was observed repeatedly, provided that the EV preparation was first pelleted to separate the

FIG. 7. Comparative SDS-acrylamide gel electropherogram of a mixture of coxsackievirus B3- ^3H -amino acids (^3Haa) and coxsackievirus B3- ^{14}C -valine preparations.

FIG. 8. Comparative SDS-acrylamide gel electropherogram of a mixture of eluted [^3H -amino acids (^3Haa)] and normal (^{14}C -valine) coxsackievirus B3 preparations.

eluted virions from the dissociated VP 4. In addition, the EV recovered from the peak fractions of the tartrate gradient (Fig. 3) contained only the three major virus polypeptides.

In the final experiment to be reported, another preparation of CAV labeled with ^3H -amino acids was prepared as previously described, and the cells were resuspended in 10 ml of buffered saline (pH 7.0) at a final concentration of 2×10^7 cells/ml for elution of virus at 37 C for 30 min. The cells were sedimented by centrifugation, and 8.0 ml of the supernatant fluid (EV) was centrifuged for 2 hr at 38,000 rev/min in an angle rotor (Spinco no. 50) to pellet the eluted virions. The supernatant fluid contained 5% of the total counts of the EV preparation. The pellet with 95% of the counts was resuspended in saline and solubilizing solution, heated, and assayed by SDS-polyacrylamide gel electrophoresis. A profile without VP 4, similar to that for EV in Fig. 8, was obtained. The supernatant fluid (7.5 ml) from the EV preparation received 0.4 ml of cold trichloroacetic acid to precipitate the protein. After 1 hr at 4 C, the precipitate was deposited by centrifugation for 10 min at $900 \times g$. Approximately 50% of the radioactivity was recovered in the trichloroacetic acid precipitate, which was taken up in 0.1 ml of saline plus 0.2 ml of solubilizing solution, heated, and assayed by gel electrophoresis. The results (Fig. 9) revealed that the radioactivity recovered from the supernatant fraction of the EV preparation migrated as VP 4. When a sample of the original EV preparation was precipitated with trichloroacetic acid before high-speed centrifugation and the precipitate was assayed by gel electrophoresis as a control of the trichloroacetic acid precipitation procedure, a typical profile of the four virus polypeptides of

native virus was obtained. Thus, we concluded that VP 4 is solubilized from the virus capsid during the process of elution, and that both VP 4 and the eluted virion devoid of VP 4 can be recovered separately by centrifugation and trichloroacetic acid precipitation procedures.

DISCUSSION

In studying the elution phenomenon of enteroviruses, we have extended the original observations of Joklik and Darnell (11) and of Fenwick and Cooper (10) for polioviruses to include another member of the group, namely, coxsackievirus B3. In the present study and in one presented elsewhere (6), we found that a large percentage of radioactively labeled and purified coxsackievirus B3 follows a pattern of attachment and elution similar to that of polioviruses. The eluted radioactivity was shown to be in the form of altered virions, which contained infectious RNA and yet were not capable of initiating infection in their altered form. The RNA labeled with ^{32}P -phosphate was resistant to hydrolysis by ribonuclease, indicating that the viral nucleic acid retained its internal position after elution. The eluted virus showed a lighter buoyant density than native virus in a potassium tartrate gradient, sedimented more slowly in sucrose gradients, and was labile when centrifuged in cesium chloride, all of which attest to its altered form. Eluted virus was also shown to be unable to reattach to freshly prepared HeLa cells. All of these changes observed in the nature of the eluted virus may reflect the loss from the capsid of a very essential low-molecular-weight protein, VP 4. Evidence presented herein suggests that VP 4 is located at the surface of normal virions and probably should not be considered as an internal protein as proposed by Maizel et al. for poliovirus (15). In recent studies, VP 4 has also been dissociated from the virions of a rhinovirus (Lonberg-Holm, *personal communication*) and from poliovirus T1 (1) without concurrent release of RNA. It is quite interesting that VP 4 may serve to stabilize the virion against inactivation by adverse environmental conditions and engender the native antigenicity (1) of the picornaviruses. It is not yet resolved whether VP 4 or VP 2 carries the attachment sites for binding with receptors of susceptible cells, but, because procapsids fail to attach to susceptible cells (*unpublished data*) and VP 4 is lost at elution, VP 2 may carry the sites binding to cellular components. Studies are in progress to determine the function of the polypeptides of coxsackievirus B3.

By using the newer method devised by Cooper, Summers, and Maizel (4) for disruption of virions

FIG. 9. Recovery of VP 4 from a preparation of eluted coxsackievirus B3- ^3H -amino acids by trichloroacetic acid precipitation as revealed by SDS-acrylamide gel electrophoresis.

for analysis by SDS-polyacrylamide gel electrophoresis, we routinely were able to obtain four polypeptides of different molecular weights from coxsackievirus B3. The electrophoretic profile for B3 virus was very similar to that of poliovirus T1, with the exception that the three major polypeptides, VP 1, 2, and 3, of B3 virus were slightly smaller than their poliovirus counterparts. The low-molecular-weight polypeptide, VP 4, from each virus migrated together in coelectrophoresis assays, but the molecular-weight assignment is uncertain in this region of the gels. At present, it is difficult to understand why fewer than four polypeptides were resolved for coxsackievirus B1 and B5 (12), whereas five were found for some poliovirus strains (4). A meaningful architecture and polypeptide composition of the virions within the enterovirus group still remain obscure.

Relatively little light has been shed upon the nature of the elution phenomenon as it relates to the infectious process for enteroviruses. The finding, however, that VP 4 is dissociated from the coxsackievirus B3 virion during elution suggests that VP 4 may be the first virion polypeptide to be released by cellular receptors in preparation for the next stage leading to productive infection. Whether in the case of eluted virus the structure of the original virions is slightly abnormal, predisposing the virus to elute, or whether features of some of the cellular receptors which bind virus are not homogenous and produce the differential pathways leading to elution or productive uncoating of the viral RNA remains to be determined.

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