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KINETIC STUDIES ON THE THERMAL DEGRADATION OF PURIFIED POLIOVIRUS[1]

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SUMMARY Analysis on poliovirus by sucrose gradient centrifugation showed that, when heated at 55°C for 30 minutes, purified polio (MAHONEY) virions with N antigenicity were converted to empty capsids with H antigenicity, which had no ribonucleic acid (RNA). The RNA-deficient capsids were found as a single peak in sedimentation and were less sedimentable than the virions. The kinetics of the release of RNA from the virions, the antigenic conversion of N to H and the loss of infectivity on heating at 45°C and 55°C were examined. It was found that the rate of RNA release from the virion was very similar to that of the antigenic conversion. Successive morphological changes of the virus particles from full particles to empty shells, on heat-treatment, were seen by electron microscopy. The results suggested a possible relationship between the mechanism of viral RNA liberation and the antigenic change in the poliovirus particles.

INTRODUCTION

Our previous studies (WATANABE *et al.*, 1965) on poliovirus gave direct evidence obtained by sucrose gradient centrifugation that heat-treatment of the virion resulted in a dissociation of viral ribonucleic acid (RNA) from the viral capsid. On the other hand, it is well known that heat-treatment causes an antigenic change of N (D) to H (C) in poliovirus particles (ROIZMAN *et al.*, 1959; HUMMELER and HAMPARIAN, 1958; LE BOUVIER, 1959). Thus it was of interest to see whether there was any correlation between the RNA liberation from the virion and the antigenic change of the viral capsid on heat-treatment.

This communication describes kinetic studies on the thermal degradation of highly purified poliovirus, with special emphasis on the relation between the structural and immunological changes of the virus particles on heat-treatment.

MATERIALS AND METHODS

1. *Virus and cells*

The Mahoney strain of poliovirus, type 1, was used throughout. This virus was not plaque-purified.

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HeLa S_3 cell monolayer cultures in Roux bottles were used for virus production. The growth medium consisted of 0.5 per cent lactalbumin hydrolyzate, 0.1 per cent yeast extract and 10 per cent unheated bovine serum in Earle's balanced salt solution.

2. *Preparation and purification of virus*

HeLa S_3 cell monolayers were infected with virus at an input multiplicity of 2 to 4 in maintenance medium containing 2 per cent heat-inactivated horse serum instead of the bovine serum used in the growth medium. Approximately 20 hours after infection, the cell sheets had been completely destroyed by cytopathic effect and then the cultures were harvested. The mixture of cells and fluid was pooled and kept in a frozen state at–20°C until use. The thawed material was homogenized in an Omni-mixer (Ivan Sorvall Inc.) at 16,000 rpm for 5 minutes, and then centrifuged at $7,550 \times g$ for 10 minutes to remove cell debris.

Furification was carried out by a modification of the method of HOYER *et al.* (1959). The bulk of the viral material was concentrated to about one fifth, using Carbowax or polyvinyl-pyrrolidone and then dialyzed against 0.01 M phosphate buffer, pH 7.1 (P.B.) for 18 to 20 hours. The concentrate was centrifuged at $100,000 \times g$ for 120 minutes (Hitachi, Type 40 P, RP 40 rotor) and the resultant pellet was resuspended in a small volume of P.B. To this suspension, a half volume of fluorocarbon (Daiflon S-3, Osaka-Kinzoku Kogyo Ltd.) was added and the mixture was homogenized in an Omni-mixer (KETLER *et al.*, 1961). This treatment was repeated 4 times. The clarified supernatant was chromatographed on a DEAE-cellulose column using P.B. as an eluent. Usually 3 to 5 ml of viral material was charged on the DEAE column (10×100 mm). Fractions of about 3 ml of eluate were collected. The eluates corresponding to the first peak of optical density (OD) at 260 mμ were pooled. The ratio of the OD at 260 to that at 280 mμ was about 1.7. This value suggests that the virus preparation is highly purified (HOYER *et al.*, 1959).

3. *Preparation of purified virus labeled with ^{32}P or ^{14}C*

Virus with RNA labeled with ^{32}P was prepared in 3×10^7 cells in monolayers in Roux bottles in 20 ml of medium consisting of 100 μc of ^{32}P-orthophosphate (Radiochemical Centre, England), 2 mM of L-glutamine and 0.25 mM each of adenosine, guanosine, uridine and cytidine in 0.005 M Tris buffered Hanks' balanced salt solution lacking phosphate, and supplemented with 2 per cent horse serum dialyzed against Tris-HCl buffer, pH 7.1. ^{14}C (protein)-labeled virus was produced by a similar procedure using Hanks' solution containing ^{14}C-chlorella protein hydrolyzate with a specific activity of 0.05 mc/0.25 mg (Radiochemical Center, England). The labeled virus materials were purified by the same procedure as non-labeled virus. Radioactivity was determined in a windowless gas flow counter. The properties of purified ^{32}P- and ^{14}C-labeled viruses are shown in Table 1.

4. *Density gradient centrifugation*

Virus samples were layered on medium consisting of 15–40 per cent sucrose in Tris-buffer. Sedimentation was carried out in Hitachi 40 P Centrifuge, using a swinging bucket rotor, at 35,000 rpm for 80 minutes. Approximately 15 fractions were obtained by puncture of the bottom of the tube.

5. *Infectivity titration*

Infectivity of virus was assayed by the plaque technique using HeLa S_3 cells. A three day-HeLa monolayer culture in a 2 ounce prescription bottle was inoculated with 0.2 ml of appropriately diluted virus. After an adsorption period of one hour, it was overlayed with nutrient agar consisting of 0.5 per cent lactalbumin, 0.1 per cent yeast extract, 5 per cent horse serum and 1 : 20,000 netural red in Earle's balanced salt solution. Two or three days after inoculation, plaques were counted and the plaque forming units (PFU) of the test virus were calculated.

6. *Titration of N and H antigens*

N and H antigens were assayed by the complement fixation (CF) test using mono-reactive N and H antisera which were prepared by guinea pig im-

TABLE 1 *Properties of purified poliovirus labeled with ^{32}P or ^{14}C*

Property	^{32}P virus	^{14}C virus
OD at 260 mμ	0.28	0.31
OD at 260 mμ : OD at 280 mμ	1.69	1.70
Plaque forming units (PFU)/ml	9.9×10^8	1.5×10^9
c.p.m./10^5 PFU	4.5	5.6
Per cent of acid-soluble counts	4	*

* Not measured

munizations (HUMMELER and TUMILOWICZ, 1960). For the CF test, 0.1 ml of an appropriate dilution of the antigen, 4 units of antiserum in 0.1 ml and 2 exact units of complement in 0.2 ml were mixed and allowed to stand for 16–20 hours at 4°C. Then 0.2 ml of amboceptor was added and the mixtures were incubated in a 37°C-water bath for about 30 minutes. Then the degree of hemolysis was measured. Veronal buffered saline containing 0.01 per cent magnesium sulfate was used as the diluent for every factor. Titers are expressed as the highest dilution of antigen giving 50 per cent fixation of complement.

7. *Assay of RNA associated with intact virion*

The procedure used was based on the fact that viral RNA within intact virions is not susceptible to RNase but is precipitated by perchloric acid (PCA). The RNA associated with intact virions was designated as RNA in virion in this paper. In contrast, the RNA dissociated from the capsids is highly susceptible to RNase so that becomes acid-soluble after treatment with RNase. Purified ^{32}P-virus was treated with 10 μg/ml of RNase in P.B. containing 0.14 M NaCl at room temperature for 60 minutes. Then 1 mg of bovine serum albumin was added as carrier and PCA was added to a final concentration of 5 per cent. The precipitate was sedimented by centrifugation, washed 3 times with cold 2 per cent PCA and then dissolved in 1 ml of 1 N NH_4OH. Protions were dried on planchets for counting. Preliminary experiments showed that less than 5 per cent of the label in unheated purified ^{32}P-virus was digested by RNase and the remaining was precipitated by PCA. On the other hand, after heating at 55°C for 30 minutes, more than 95 per cent of the label was digested to acid-soluble materials by RNase.

8. *Heat-treatment of virus*

Samples were placed directly in the bottom of tubes, and the tubes were sealed with a rubber stopper. All the test tubes used were of uniform size and thickness (13 × 100 mm, 1.2 mm wall thickness). The tubes were placed in a water bath at the desired temperature, to a depth of 2 cm below the surface. At the end of the heating period, the tubes were quickly transfered to an ice bath.

9. *Electron microscopy*

The nagative staining method (BRENNER and HORNE, 1959) was employed. Virus materials were mixed with an equal volume of 2 per cent phosphotungstic acid of pH 7.0. Mixtures were placed on carbon coated grids. The preparations were examined in a Hitachi HU-11A electron microscope at an instrumental magnification of 4×10^4.

RESULTS

1. *Fractionation of unheated and heated virus by sucrose gradient centrifugation*

It was reported previously (WATANABE *et al.* 1965) that heat-treatment of poliovirus for 15 minutes at 56°C resulted in the dissociation of RNA from the viral capasids. However, the infectivity and N and H antigens in the fractions were not assayed at that time. To study the relationship between the changes in antigenicity, infectivity and sedimentation properties of the heated virus preparation, pruified virus labeled with either ^{32}P or ^{14}C, which had been heated at 55°C for 30 minutes were fractionated by sucrose gradient centrifugation, comparing the results with those on unheated virus.

The results are shown in Fig. 1. The unheated virus gave a single peak of label which coincided with those of infectivity and N antigenicity, as seen in Figs. 1-a and -c. The results suggest that the virus preparation used was highly purified. However, as seen in Fig. 1-b, the heated preparation of ^{32}P-labeled virus showed a single peak of H antigen which sedimented slower than that of the unheated virus. The radioactivity was distributed in less sedimentable fractions than H antigen. This suggested that the ^{32}P-labeled viral RNA separated from the viral capsid with H antigenicity, on heating the virion. As shown in Fig. 1-d, the heated preparation of virus labeled with ^{14}C-amino acids gave a single peak of H antigen which coincided with that of radioactivity and this peak sedimented slower than that of unheated virus. The infectivity and N antigenicity of each fraction in heated preparations, were not measured, because the infectivity was reduced to less than 0.01 per cent of that of the unheated virus and no N antigenicity was detectable in these preparations, before sedimentation.

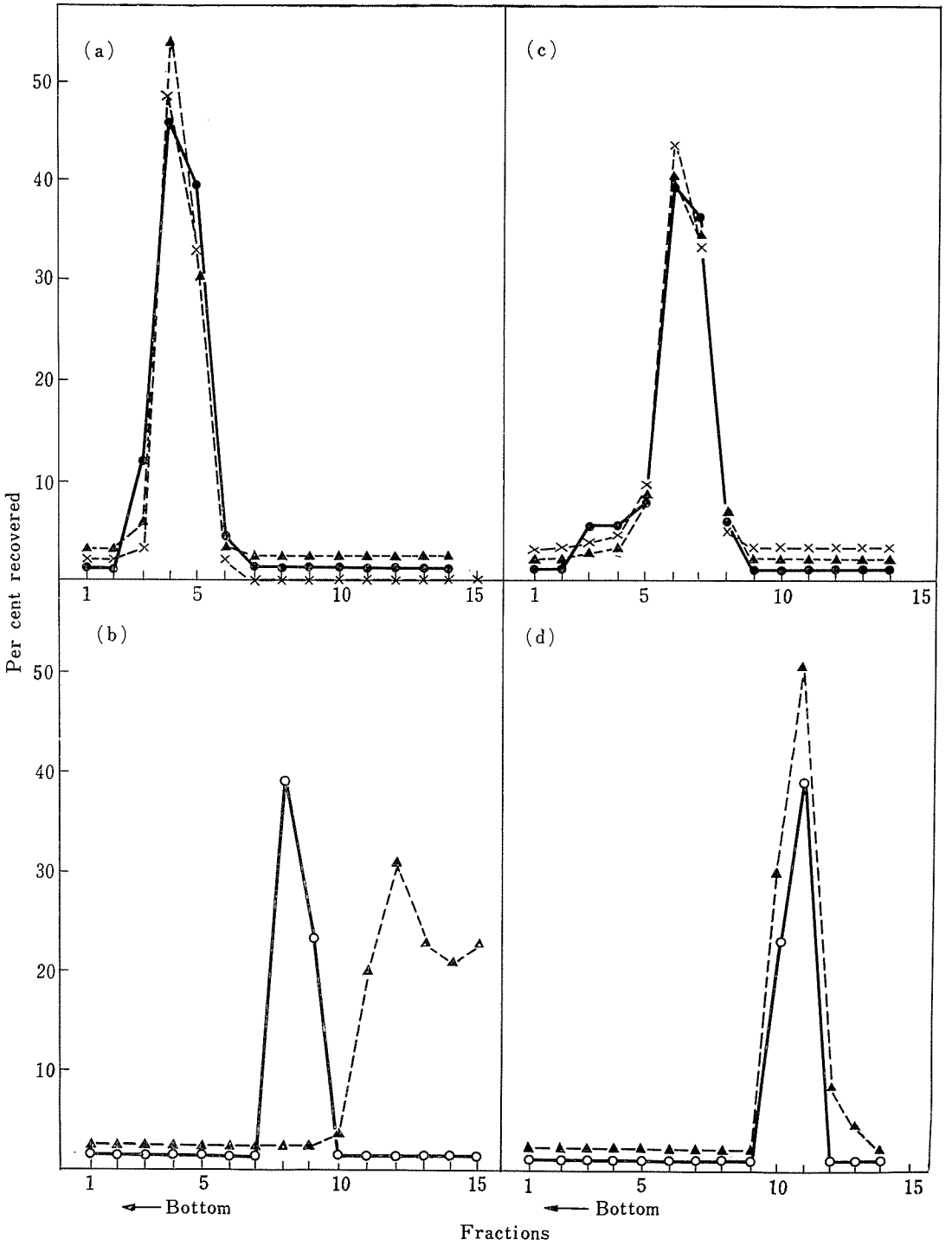
FIGURE 1 Sucrose gradient sedimentation of purified viruses labeled with ^{32}P or ^{14}C before and after heating at 55°C for 30 minutes. The viruses used were the same as those shown in Table 1.

(a) ^{32}P virus before heating (c) ^{14}C virus before heating

(b) ^{32}P virus after heating (d) ^{14}C virus after heating

●——● N antigen ▲- - - - -▲ Radioactivity

○——○ H antigen ×·····× Infectivity


(a)
(c)
(b)
(d)
Per cent recovered
50
40
30
20
10
1
5
10
15
Bottom
Fractions

2. *Effect of various temperatures on the degradation of virus*

Heat-treatment of ^{32}P-virus suspended in 0.01 M phosphate buffer containing 0.14 M NaCl (PBS) was carried out at various temperatures between 0° to 55°C for periods of 10 minutes, and the titers of both the N and H antigens and the ^{32}P-RNA in the virion were assayed.

A typical result is shown in Table 2. Complete antigenic conversion, in terms of the level detectable by the CF test, occurred by 50°C. Heating at 50°C also resulted in the release of more than 90 per cent of the RNA. The results indicate that at a higher temperature both RNA-release and antigenic conversion are rapid and that the two reactions proceed at roughly the same rate. This parallelism between the rates of RNA-release and antigenic conversion was constantly observed in duplicate experiments.

3. *Kinetics of the changes in the properties of virus on heating*

Two samples of purified ^{32}P-virus preparation in PBS were heated at 55°C and at 45°C, respectively. Samples were withdrawn at intervals, chilled rapidly and their CF reactivities against N and H antisera, radioactivity of ^{32}P-RNA in the virion and infectivity were measured.

TABLE 2 *Effect of heat-treatment on the rates of RNA release and antigenic conversion. Purified ^{32}P virus suspended in 0.01 M phosphate buffer containing 0.14 M NaCl was heated at the indicated temperatures for 10 minutes*

Temperature	CF antigen units/0.1 ml		^{32}P in virion	
	N	H	c.p.m.	%
0°C	16	<4	1983	100
35	16	<4	1416	71
45	4	8	306	12
50	<4	16	38	2
55	<4	16	26	1

As shown in Fig. 2-a, heating at 55°C altered the antigenic specificity; the amount of N antigen decreased during the heat-treatment and concomitantly H antigen increased. This antigenic conversion was complete within 3 minutes. Loss of both RNA in the virion and infectivity were also seen during this treatment. The rates of these reductions were the same as the rate of antigenic conversion. Heat-treatment at 55°C did not seem suitable for comparison of the kinetics of the changes in these properties of the virus, since at this temperature these changes were so rapid that they were more than 90 per cent complete within 3 minutes.

Upon heating at 45°C, much slower changes in these parameters were observed, as shown in Fig. 2-b. The reduction curves of the N antigen was almost a mirror image of the increment of H antigen. The curves of reduction of ^{32}P-RNA in the virion and of N antigen were similar. The antigenic conversion and RNA extrusion were also accompanied by loss of infectivity. However, the rate of reduction of infectivity was not always in parallel with that of N antigen or ^{32}P-RNA in virion, the former sometimes being slower or faster than the latter reductions.

These results strongly suggest that the RNA-release from the virion is closely associated with the mechanism of antigenic conversion of N to H, under the present conditions.

4. *Electron microscopic examination of heated virus*

A comparison of the morphological changes of heated and unheated virus was made by electron microscopy, using negatively stained preparations. The virus eluted from the DEAE-cellulose column in the step of purification was kept at 0–4°C and examined within 2–5 hours.

Samples of fresh unheated virus mostly appeared as full particles, as shown in Fig. 3. However, a few empty particles were also seen.

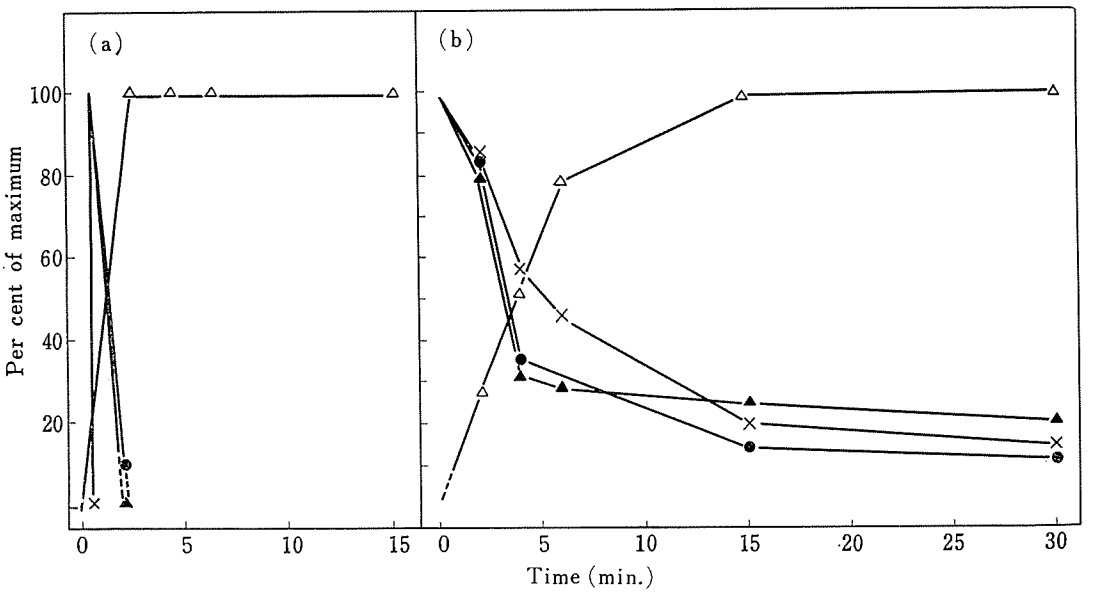


FIGURE 2 Kinetics of the antigenic conversion, loss of infectivity and reduction of ^{32}P in virion during heat-treatment at 55°C (a) and at 45°C (b).

△——△ H CF antigen ▲——▲ N CF antigen

×——× PFU ●——● ^{32}P precipitated with PCA

On heating at 45°C or 55°C successive morphological changes of the virus particles were observed. Virus preparation which had been heated at 45°C for 10 minutes is illustrated in Fig. 4. This preparation characteristically consisted of a mixture of full particles, empty particles and particles with irregular forms. Fig. 5 shows a virus preparation which had been heated at 55°C for 30 minutes. Most of the virus particles are empty shells of a regular shape but some particles of irregular form are also seen, though not so many as in the preparation heated at 45°C.

These results could be interpreted as due to the fact that the heat-treatment causes successive morphological changes of virus particles leading to the formation of empty shells lacking a core substance. This observation is quite consistent with the results of kinetic studies on RNA-release, reduction in infectivity and change in antigenicity described in the preceding section.

DISCUSSION

In earlier studies by ROIZMAN *et al.* (1959), it was reported that the identity of a heat denatured antigen with a naturally occuring C (H) antigen of poliovirus could not be proved, since the heated virus reacting with C (H) antibody was not located in zone corresponding to that of natural H antigen on a sucrose gradient centrifugation. They suggested that heating the virus at 56°C might lead to an aggregation of particles in addition to the immunological change. In a previous paper (WATANABE *et al.* 1965), however, using ^{14}C-labeled virus, the authors clearly showed that heated virus particles sedimented at the same rate as naturally occuring empty shells and at a different rate from the virion particles. The experiments described here demonstrated that purified ^{14}C-(protein)-labeled virus particles with a sedimentation constant of 160 S, and with N antigenicity and infectivity are converted to non-

infectious 80 S particles with H antigenicity but without N antigenicity by heat-treatment at 55°C for 30 minutes. The empty shells of the 80 S particles produced by heat-treatment were clearly seen by electron microscopy. The observed dissociation of the viral RNA from the viral capsid, together with the results of immunological and morphological examinations lead to the following tentative conclusion. When intact virion is heated, the viral RNA is released from the particles and the N antigenicity of the virion is simultaneously converted to H antigenicity of the empty shell. The empty shell is probably composed of viral protein which is dissociated from the viral RNA. Furthermore, the intact virion has only N antigenicity, but the empty particles, whether naturally occurring or produced by heat-treatment, always possess H antigenicity.

Kinetic studies on the thermal degradation of poliovirus, measured by biological, immunological and physical characters, especially during heat-treatment at 45°C, showed a close correlation between the rate of antigenic conversion of N to H and the rate of RNA release from the virion. The observed successive morphological changes of full particles to empty shells during heat-treatment, as revealed by electron microscopy, support the successive liberation of core RNA from the virus particle. These facts suggest a causal relationship between the mechanisms of viral RNA-liberation and the antigenic conversion, although it is possible they may be independent and it is just a coincidence that they happened to occur at similar rates. It seems probable that the N antigenicity is determined by a particular configuration of the protein which is caused by its specific interaction with viral RNA located inside the viral capsid. The H-antigenic determinant is only found in the RNA-free viral capsid configurated as a particle and not in disrupted subunits, as shown in previous reports (WATANABE *et al*, 1965; SCHARFF *et al.*, 1964). If this is so, it is possible that the dissocation of RNA from the capsid causes the configurational change in viral protein which causes the antigenic conversion of N to H. The mechanism for this latter might be that the H antigenic determinant appears in the capsid protein due to an alteration of its secondary structure, possibly caused by extrusion of RNA. Another possibility is that denaturation or a configurational change of the capsid protein corresponding to the antigenic change of N to H is the primary event which triggers off the release of RNA from the particle.

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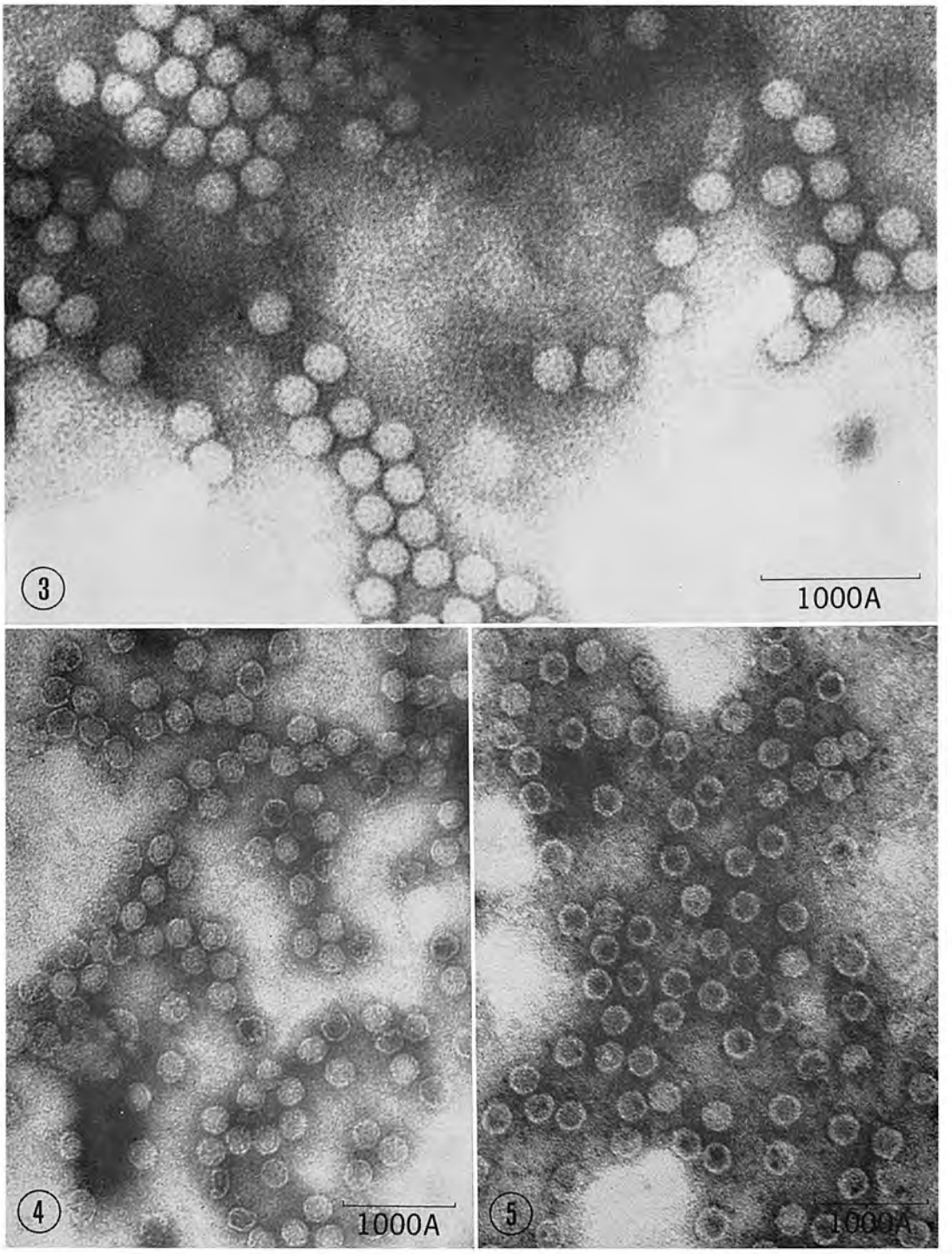
FIGURE 3 Fresh unheated virus.

FIGURE 4 Virus heated at 45°C for 10 minutes.

FIGURE 5 Virus heated at 55°C for 30 minutes.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