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Biophysical and immunological studies on the differential effect of guanidine hydrochloride on type A and type B influenza viruses

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SUMMARY

Guanidine hydrochloride selectively inactivated both the biological activity and the immunogenicity of the haemagglutinin of influenza A/X-7 (H0N2). The residual neuraminidase was fully active biologically and immunologically. The reverse was observed with influenza B/ROB; with this virus the haemagglutinin was resistant, and was immunogenic; while the neuraminidase was selectively inactivated, and was not immunogenic.

INTRODUCTION

In a previous article a differential effect of guanidine hydrochloride (GH) on over 30 different strains of influenza virus was reported (David-West & Belyavin, 1973). It was shown that generally the neuraminidase (Nase) of the type A viruses, especially the 1957–68 (H2N2) or H3N2 strains, was resistant to GH inactivation, while the haemagglutinin (HA) was sensitive. On the other hand, with the type B viruses the Nase was sensitive, while the HA was relatively resistant. The results of experiments with recombinant strains of influenza viruses suggested the possible application of GH resistance or sensitivity as a marker for the virus-coded surface subunits (David-West, 1973). As a further exploration of the differential effect of GH on the surface subunits of the two groups of influenza viruses some correlative biophysical and immunological studies were conducted. The purpose of these studies is at least twofold; first to provide a method for the selective production of antibodies against either of the subunits, after the selective inactivation by GH. Secondly, GH attacks polar linkages such as hydrogen bonds; thus its effect would elucidate the significance of such bonding in the immunogenically active sites of the Nase and HA. Both of these would add to existing knowledge on the structure and immunogenicity of the Nase and the HA in general, and in particular highlight some other basic differences between influenza virus types A and B.

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MATERIALS AND METHODS

Viruses

Influenza A/X-7 (F1), a recombinant of A/NWS/33 and the A/R15+ strain of Jap/57, having the Nase (N2) of the 1957 parent, and the HA (H0) of the 1933 parent (Kilbourne *et al.* 1967); and influenza B/ROB/55.

Propagation and purification of virus

The methods were as previously described (David-West & Belyavin, 1973). The essential steps were as follows: Virus was propagated in the allantoic cavity of 10- or 11-day old embryonated hens eggs. The infective fluid was harvested after 72 hr. at 35° C. After clarification in a bench centrifuge the virus was pelleted at 90,000*g* for 1 hr. The pellet was taken up in minimum amount of 0·15 M-NaCl + 0·01 M Tris-HCl pH 7·5 (Tris-saline), and after some few minutes on a Vortex mixer in order to disaggregate the virus, a further clarification was carried out as before. Finally the virus was purified by rate zonal centrifugation through a linear (10–50%, w/v) sodium glutamate gradient in Tris-saline at 65,000*g* for 45 min. in a Spinco Model L ultracentrifuge, using rotor SW 39. Fractions were collected and titrated for HA activity. The peak fractions were pooled, dialysed against Tris-saline in the cold, and used as virus stock for subsequent studies.

Isotope-labelled virus was prepared by incorporating [^{14}C]protein hydrolysate (52 mc./m-atom carbon) into the inoculum. Each egg received 5 μc. of the isotope. The ultimate processing of the infective fluid was as described earlier. The isotope was procured from the Radiochemical Centre, Amersham, England. Measurement of radioactivity of purified virus used in the appropriate isopycnic centrifugation experiments was performed in a Packard Tri-Carb scintillation counter. The scintillation fluid consisted of a mixture of toluene-methanol-Cab-O-sil.

Guanidine treatment

A stock solution of guanidine hydrochloride (Grade 1; Sigma) was prepared in Tris-saline. Virus preparation and guanidine stock solution were mixed to give final molar concentration of either 2·0 or 4·0 M. Reaction was at 37° C. for 30 min. In the control Tris-saline, at the same pH, replaced guanidine. A few experiments were also performed with urea, for comparison.

Isopycnic centrifugation of treated or control preparations was carried out at 100,000*g* for 4 hr., through linear 10–50% sodium glutamate gradient in 3 × 5 ml. swing-out rotor in Spinco Model L, using the SW 39 rotor.

Immunization of animals

White New Zealand rabbits were immunized with 2 M guanidine-treated or untreated virus. Each inoculation consisted of 0·4 ml. given by the ear vein. The schedules of inoculation are shown in the appropriate figures (Figs. 2, 3). The starting virus concentration was 40,000 haemagglutinating (HA) units per ml.

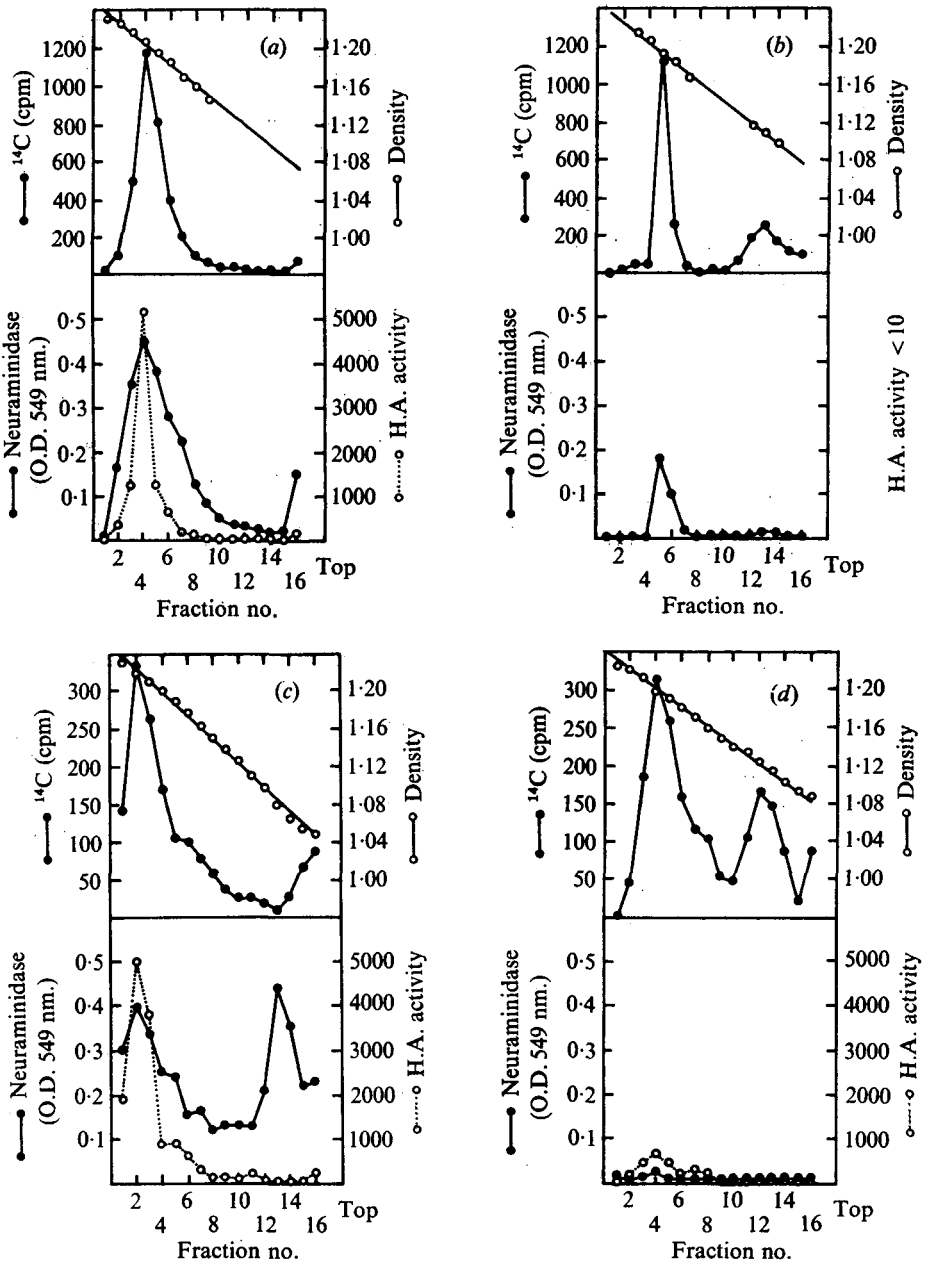

Fig. 1(*a*). Isopycnic centrifugation of untreated A/X-7. (*b*) Isopycnic centrifugation of 4 M guanidine-treated A/X-7. (*c*) Isopycnic centrifugation of untreated B/ROB. (Note that there is some splitting off of Nase.) (*d*) Isopycnic centrifugation of 4 M guanidine treated B/ROB.

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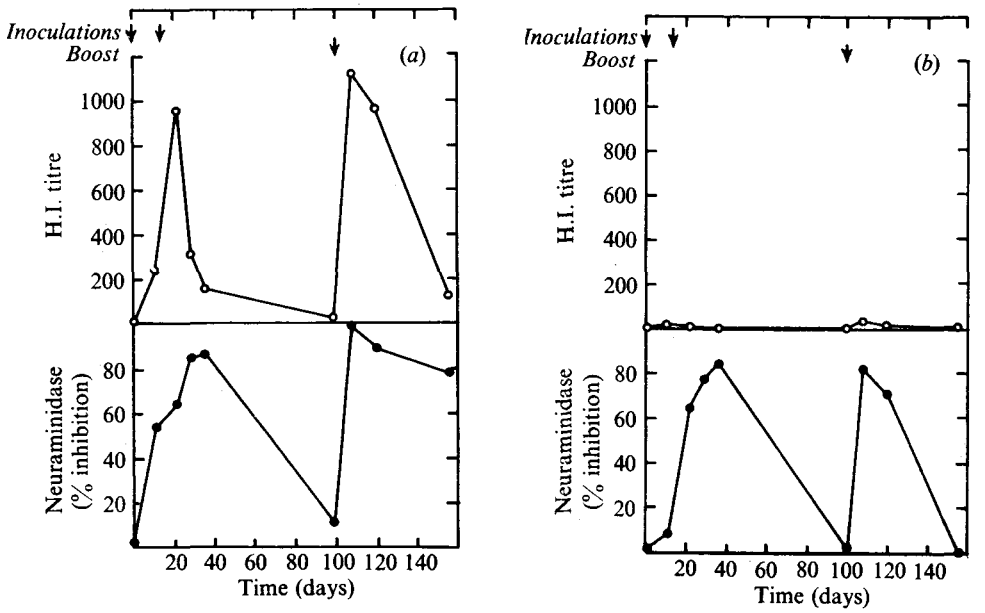

Fig. 2(*a*). Primary and secondary antibody induction for the haemagglutinin or the neuraminidase of untreated A/X-7. (*b*) Selective induction of anti-neuraminidase antibody by 2 M guanidine-treated A/X-7. Immunization schedule: two initial injections spaced 2 weeks apart, followed by a booster dose after 3 months.

for virus X-7, and 20,000 HA units per ml. for virus B/ROB. After treatment with guanidine there was no detectable residual HA activity with the X-7 virus, while the B/ROB virus still had 5000 HA units per ml.

Haemagglutination test

This was done by the micro-titre method using Takátsy loops. Serial twofold dilutions of test material were prepared in Tris-saline, and equal volumes of freshly prepared 0·5% fowl red blood cell suspension were added. The test was read after 45 min. at room temperature.

Haemagglutination-inhibition test

The test sera were inactivated at 56° C. for 30 min. and later periodated. One volume of serum was treated with 3 vols. of 0·9 M sodium periodate in Tris-saline. The reaction was allowed to continue for 15 min. at 4° C. in the dark, and was stopped by the addition of 1 vol. 10% glucose-saline. Four HA units of purified virus was used.

Neutralization test

Serial twofold dilutions of serum were mixed with equal volumes of virus dilution containing 100 EID50. The mixture was incubated at 37° C. for 1 hr. Eleven-day-old embryonated eggs were inoculated with the mixture, using four eggs per dilution. Infectivity was determined by HA after 48 hr. at 37° C.

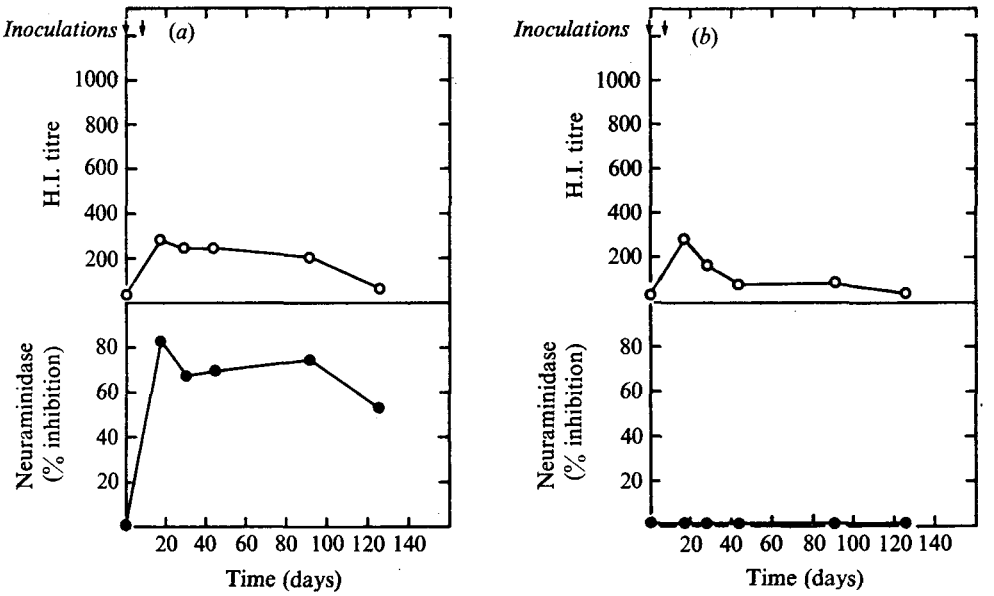

Fig. 3(*a*). Primary antibody induction for the haemagglutinin or the neuraminidase of untreated B/ROB. (*b*) Selective induction of anti-haemagglutinin antibody by 2 M guanidine-treated B/ROB. Immunization schedule: two injections given 1 week apart. No booster dose.

Neuraminidase assay

Test material and collocalia mucoid substrate were mixed and incubated overnight at 37° C. The substrate was prepared in 0·02 M sodium acetate buffer in 0·15 M sodium chloride, pH 6·0 (Kendal, Biddle & Belyavin, 1968). Free sialic acid was determined by the method of Aminoff (1961). All tests were set up in duplicates.

Electron microscopy

Virus preparations (guanidine-treated or control) were fixed in 3·0% glutaraldehyde in phosphate buffer at neutral pH. These were negatively stained with 3·0% ammonium molybdate, pH 6·5, and examined in the Philips EM 300 electron microscope.

RESULTS

Isopycnic banding of labelled virus

Fig. 1(*a*) and (*b*) show the distribution of viral activities in various fractions of control X-7 and 4 M guanidine-treated X-7, after density-gradient centrifugation. In the control preparation there were coincident single peaks of HA, Nase and radioactivity in fraction 4, with a density of 1·207 g. cm.^{3}. In the treated preparation HA activity was reduced to below detection, while a substantial amount of the Nase still remained. The peak of enzyme activity was shifted to fraction 5, with a density of 1·195 g./cm.^{3}. Another set of experiments with 2 M guanidine or 4 M urea showed that with either treatment the HA

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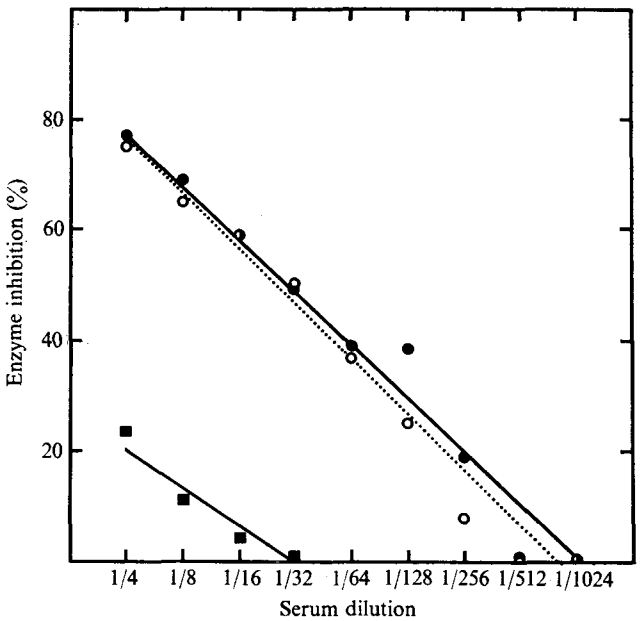

Fig. 4. Comparative neuraminidase-inhibition test with antiserum produced against untreated A/X-7 and antiserum against guanidine-treated A/X-7. Note: serum prepared without adjuvant. X-7: ○.....○, control; ●—●, 2 M guanidine. ■—■, Normal rabbit serum.

activity was completely obliterated, while the residual Nase activity varied between 80% and 90% in independent experiments.

In the B/ROB experiments the control virus preparation had coincident single peaks of HA, Nase and radioactivity in fraction 2, with a density of 1·220 g. cm.^{3}. After 4 M guanidine treatment the Nase activity was completely destroyed while about 13% HA activity remained (Fig. 1*c*, *d*) with a density of 1·198 g. cm.^{3}. The figures also show that there was a certain amount of splitting of B/ROB during the process of ultracentrifugation, as evidenced by the appearance of 'soluble' Nase activity in the top fraction of the gradient (Fig. 1*c*). This split-off enzyme activity is, however, also sensitive to guanidine as was that of the major coincident activities (HA, Nase and radioactivity) peak of fraction 2 (Fig. 1*d*). Treatment with 2 M guanidine also completely destroyed Nase activity, but the residual HA activity was between 25% and 40%, in separate experiments.

Immunization studies

Because the differential inactivation of either HA or Nase in both viruses by either 2 M or 4 M guanidine was similar, the lower molar concentration was selected in the preparation of inoculum for immunization, since this produced greater residual activity. The results obtained with A/X-7 are shown in Fig. 2(*a*) and 2(*b*); those of B/ROB are shown in Fig. 3(*a*) and 3(*b*). Guanidine treated A/X-7 selectively induced anti-Nase antibody (Fig. 2*b*) while guanidine treated B/ROB selectively induced anti-HA antibody (Fig. 3*b*).

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The results of neutralization tests conducted with the antiserum produced by both guanidine-treated viruses showed that 1/1000 dilution of the B/ROB antiserum neutralized the infectivity of the virus in eggs. On the other hand, even 1/10 dilution of the A/X-7 antiserum did not reduce the infectivity of A/X-7 in eggs.

A comparison of the potency of the anti-Nase antibody induced by guanidine treated A/X-7 and that of the control preparation was made by testing the antiserum obtained after 35 days immunization, which was the peak of primary antibody induction. Fig. 4 shows that both are equally potent, in enzyme-inhibition titration.

Morphology of guanidine treated virus

These are shown in Plate 1. All micrographs except the last of each set (*c*, *f*) were prepared from virus treated under standard conditions of guanidine inactivation (i.e. 2 M for 30 min. at 37° C.). The last micrographs were prepared from standard preparations that had been at 4° C. for 5 days.

Both viruses retained their basic morphology after standard guanidine treatment (*b*, *e*). However, the surface spikes on these particles were more sparse and somewhat disorganized. There was also some suggestion that the treated particles had lost at least part of their internal components. This was more obvious after prolonged treatment (*c*, *f*). These figures also show that such prolonged treatment did not significantly alter the A/X-7 particles further, but the B/ROB particles, under the same conditions, were virtually 'shaved' of all surface spikes, and the particles are very much bloated.

DISCUSSION

Until recently surveillance of influenza virus infection was based primarily on the antibody directed against the haemagglutinin (anti-HA antibody). However, the virus also contains another surface subunit, the neuraminidase (Nase) which also participates in the immune response, by the induction of anti-neuraminidase (anti-Nase) antibody. These two virus-coded surface glycoproteins, the HA and the Nase, are immunologically, functionally and structurally distinct (Laver & Kilbourne, 1966; Laver & Valentine, 1969) and also undergo independent variation (Schulman & Kilbourne, 1969). The new system of nomenclature of the Type A influenza viruses proposed by the World Health Organization Expert Committee (1971) therefore took cognizance of the immunologic and genetic independence of the HA and the Nase. The Nase of the type B influenza viruses, however, did not vary significantly one from the other to justify subgrouping (Chakraverty, 1972). Unlike anti-HA antibody, anti-Nase antibody does not neutralize virus infectivity; but it does modify the course of the infection in both man and animals (Coleman *et al.* 1968; Schulman, Khakpour & Kilbourne, 1968; Schulman & Kilbourne, 1969; Schulman, 1969; Slepushkin *et al.* 1971; Allan, Madeley & Kendal, 1971; Murphy, Julius & Chanock, 1972). The modifying effect of anti-Nase antibody might play an important role in the establishment or spread of the virus in human infection (Coleman *et al.* 1968; Schulman & Kilbourne, 1969).

The maintenance of adequate surveillance of anti-Nase antibody necessarily

requires the use of virus preparations in which the HA activity is obliterated, since it is widely observed that anti-HA antibody can interfere nonspecifically with Nase activity by steric hindrance. Earlier workers have achieved this by working with monovalent Nase prepared by disrupting the virus with various detergents followed by electrophoresis, and ultimate isolation of the enzyme activity (Kilbourne, Laver, Schulman & Webster, 1968). In a previous study (David-West & Belyavin, 1973) it is shown that guanidine hydrochloride selectively inactivated the HA of type A influenza viruses, especially the H2N2 or the H3N2 strains, which also are the most frequent cause of influenza epidemics at present. But the selective inactivation of the biological activity of the HA subunit might not necessarily also reflect a concomitant inactivation of the immunogenity of the molecule, since it is conceivable that the sites of biological activity and immunologic property might occupy different loci on the molecule. Alternatively the molecular groupings for both activities might differ. The results of the present investigation have shown that, with the inactivation of the HA activity of influenza virus A/X-7 by guanidine, the immunogenicity of the molecule is also concurrently destroyed. It should also be mentioned that the guanidine-treated A/X-7 preparation was negative for monovalent or 'soluble' HA in antibody-blocking test (David-West & Belyavin, 1973). This would suggest that the action of guanidine on this surface glycoprotein is not one of solubilization as is widely reported for either detergent or proteolytic enzyme disruption, but one of *in situ* inactivation. This permitted the selective production of Nase fully active biologically and immunologically, and the antiserum prepared against the treated virus was devoid of demonstrable anti-HA activity. The reverse was observed with influenza virus B/ROB; with this virus the Nase was found to be sensitive to guanidine inactivation while the HA was relatively resistant. Furthermore, the guanidine-treated B/ROB induced the production of only anti-HA antibody in rabbits. Ultracentrifugation studies correlated with electron microscopy also showed that the residual Nase or HA in the case of B/ROB was associated with the virus particle, and was thus necessarily polyvalent. The method described here is clearly much simpler than the one involving detergent disruption and electrophoresis, and so provides a simple method of producing anti-HA or anti-Nase antibodies. The guanidine inactivation method also revealed significant basic differences between the HA and the Nase of the type A 2 and the type B test viruses. Guanidine uncouples non-covalent bonds (hydrogen bonds); the results therefore emphasize the relative importance of such bonds in the biological and immunological properties of the surface glycoproteins of the two types of influenza viruses.

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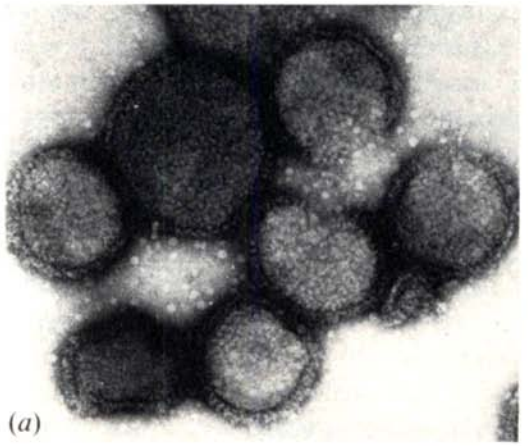
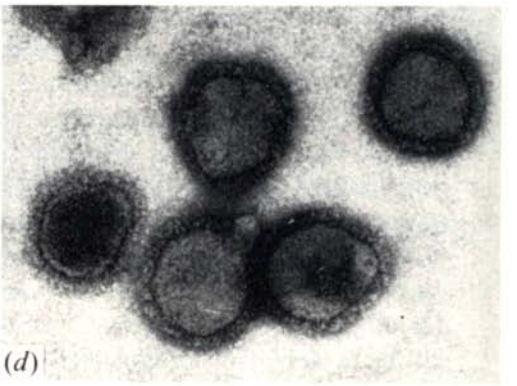

(*a*)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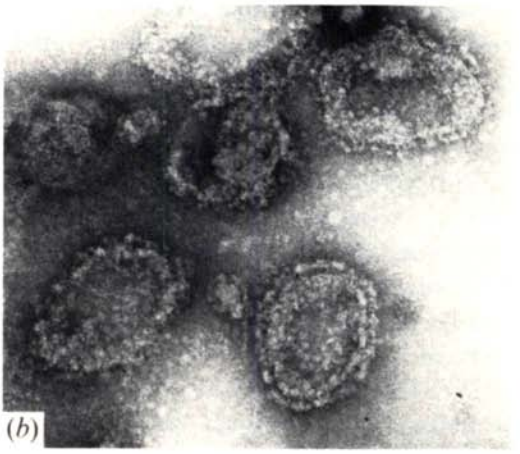
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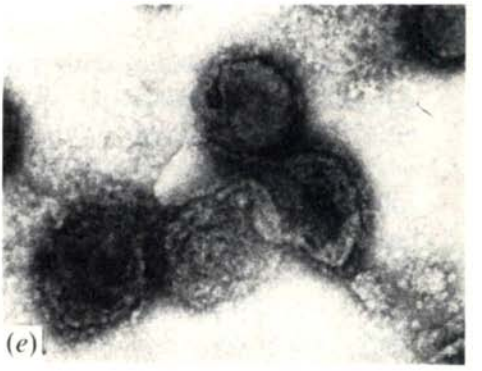
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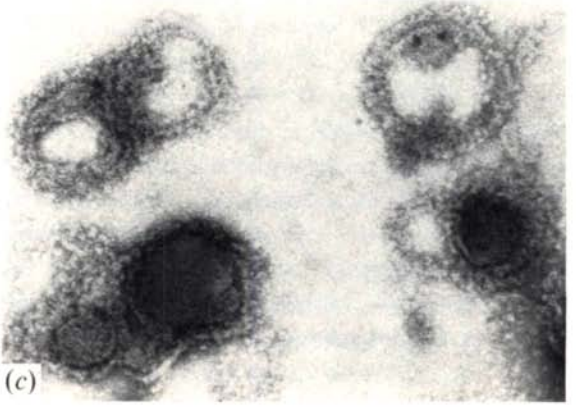
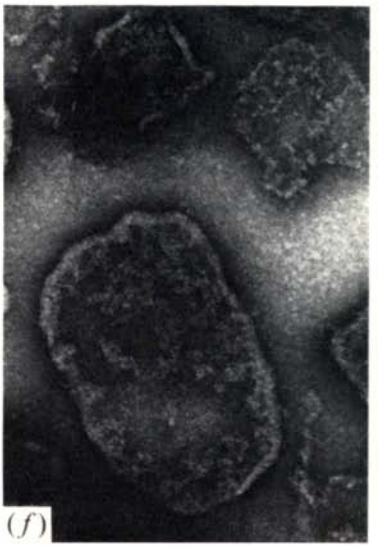

(*c*)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EXPLANATION OF PLATE

Electron-micrographs of control and guanidine-treated influenza viruses.

(*a*) Control A/X-7.

(*b*) A/X-7 treated with 2 M guanidine for 30 min. at 37° C.

(*c*) A/X-7 treated with 2 M guanidine for 5 days at 4° C.

(*d*) Control B/ROB.

(*e*) B/ROB treated with 2 M guanidine for 30 min. at 37° C.

(*f*) B/ROB treated with 2 M guanidine for 5 days at 4° C.

Magnification of each: × 160,000.