Polyethylene glycol purification of influenza virus with respect to aggregation and antigenicity

By A. POLSON, A. KEEN, C. SINCLAIR-SMITH,

M.R.C. and U.C.T. Virus Research Unit,
Medical School, Observatory, Cape Town

AND I. G. S. FURMINGER

Evans Medical Limited, Speke, Liverpool

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SUMMARY

Influenza virus may be precipitated and aggregated by polyethylene glycol into clusters comprising ten to many hundred virions per aggregate. These aggregates are sparingly soluble and may be freed of contaminating polymer by washing in the appropriate buffer at room temperature or by precipitation in 30% (v/v) ethanol at subzero temperatures.

Immunogenic studies in guinea-pigs of the virus in different states of dispersion revealed that the aggregated virus is the superior antigen to the virus in the monomeric form or in the dissociated state following treatment with ether.

INTRODUCTION

It is well known that when an animal is injected with an antigen which is in a state from which it is liberated slowly (i.e. from Freund's incomplete adjuvant) the immune response of the animal is better than when injected with the antigen in its unbound or monomeric form. Influenza virus may be aggregated by polyethylene glycol (P.E.G.) M.W. 6000 into sparingly soluble clusters containing variable numbers of virions. The antigenicity of these aggregates was investigated and compared with that of the virus in its monomeric state and also when the virus was dissolved by ether.

It was observed that influenza virus could be precipitated from suspension by P.E.G. at appreciably lower polymer concentration than found by Kanarek & Tribe (1967). As high concentrations of the polymer in influenza vaccines would not meet the approval of Governmental Health agencies it stands to reason that if the content of the polymer is at a low initial value in the precipitate, removal of traces from vaccines would be more complete and more easily achieved. This study was also undertaken to explore the ways of removing this contaminant from viral concentrates.

MATERIALS AND METHODS

Virus

Two strains were used in this work, namely (1) Hong Kong A 2/8/68, a strain originally obtained from the World Health Organization but passaged repeatedly in eggs in this laboratory, and (2) X 31 influenza strain, the genetic recombinant of a Hong Kong strain crossed with a PR 8 strain. These viral strains were grown in allantoic cavities of 10- to 14-day pre-incubated fertile hens' eggs and the infected fluid was harvested on the second day after inoculation.

Polyethylene glycol

Polyethylene glycol of molecular weight 6000 was used in preference to the lower or higher M.W. polymer as it has a favourable concentration–viscosity ratio (Polson *et al.* 1964). It was supplied by [Shell Chemicals, Cape Town. It may be added directly to the allantoic fluid as a dry powder or as a concentrated auto-claved solution (40 % by wt. in water) and was allowed to act for 1 hr. at room temperature.

Centrifuges and rotors

An MSE refrigerated centrifuge was used for freeing the infected allantoic fluid of cells and cellular debris. The No. 30 rotor and in some cases the No. 40 rotor of the Spinco preparative centrifuge Model L was employed for concentration of the finely dispersed viral aggregates. The 'thin-layer rotor' described by Polson & Stannard (1970) was employed for preparation of influenza virions in the mono-meric state in a concentrated form, freed of most of the extraneous protein. (For the purpose of this paper, spontaneous loose viral aggregation is disregarded in the use of the term 'monomeric state'.)

Analytical ultracentrifuge

Several of the preparations, namely the influenza virus obtained by thin-layer rotor centrifugation and fractions precipitated by 4 % P.E.G., were centrifuged in a Spinco Model E ultracentrifuge to establish whether the majority of the virus particles were present as monomers or as aggregates.

Electron microscope

The various influenza virus preparations were negatively stained with phospho-tungstic acid and examined in a Siemens Elmishop Model 1 A.

Antigenicity tests in guinea-pigs

Comparable groups of six guinea-pigs were used for testing each of the antigen preparations. The animals received a single intramuscular injection of formalde-hyde-treated virus material without adjuvant and were bled by heart puncture before inoculation and at 3 and 7 weeks after inoculation. Antigenic potency was assessed by haemagglutination inhibition tests with fowl red blood cells, using four haemagglutinating doses of virus and reading a 100 % end-point after 1 hr. at room temperature in a microtitration system.

EXPERIMENTAL

A. *Effect of storage and* P.E.G. *concentration on precipitation of virus*

After storage at 4° C. for 20 hr. the infected allantoic fluid was subdivided into eight equal portions, each of which was treated with an appropriate weight of P.E.G. powder. They were then centrifuged at 500–800g for 20 min. Portions of the supernatant fluids were then re-centrifuged at 1500–1900g for the same time. Samples of all the supernatants were kept and assayed for haemagglutinin content.

The above experiment was repeated in parallel upon infected allantoic fluid from the same original pool but which had been stored at 37° C. for 20 hr. The object of this test was to investigate the effect of storage at 37° C. upon the pre-cipitability of the haemagglutinin by P.E.G. If there was an appreciable amount of free haemagglutinin resulting from dissociation of virus, these smaller particles would fail to precipitate at concentrations of P.E.G. which would precipitate the intact large virus particles (Juckes, 1971).

It was established that precipitation with 2 % P.E.G. followed by centrifugation at 1500g for 20 min. would remove approximately 90 % of the H.A. activity, and several experiments were then conducted to determine the yield of the haemag-glutinating virus. This was done by repeated precipitation of the resuspended virus precipitate from diminishing volumes of dispersion medium.

Prior treatment of clarified infected allantoic fluid with 0·1 % formaldehyde did not materially alter the removal of haemagglutinin with P.E.G.

B. *Sequential precipitation with* 0·6 % *and* 4·0 % P.E.G.

The infected allantoic fluid was treated with 0·6 % P.E.G. and the resulting faint precipitate was centrifuged out of suspension at 1500–1900g for 20 min. and examined in the electron microscope. The concentration of P.E.G. in the super-natant fluid was then increased to 4 % and the new precipitate was centrifuged out and examined as before. The angle head of the preparative Spinco centrifuge was used and it was found that the virus precipitate failed to form a pellet but adhered to the surface of the centrifuge tubes remote from the centre of rotation. The material on the wall was also subjected to electron microscopy. The object of this experiment was to establish if it was possible to separate filamentous and giant forms from the smaller round or 'doughnut' forms of the virus or to eliminate undesirable material such as mycoplasma-like bodies and coarse non-specific proteinaceous substances from the virus suspension.

C. *Thin-layer rotor concentration*

The virus was concentrated in the thin-layer rotor at 1200–1400g for 60 min. The virus contained in 40 ml. infected allantoic fluid was sedimented to the extent of 90 % or more into approximately 1 ml. of fluid. This was estimated by loss of haemagglutinating activity in the supernatant fluid (SNF).

On 'washing' the virus by diluting with phosphate-buffered saline (PBS) at

pH 7·3 and a second cycle of centrifugation the associated proteins were reduced well below the level at which they could be detected by ultracentrifugation in the Model E.

D. *Antigenicity of Hong Kong A 2/8/68 virus precipitated by* P.E.G. *from infected allantoic fluid*

Thirty ml. of allantoic fluid infected with the strain of Hong Kong virus was treated with 4% P.E.G. added as a 40% autoclaved solution and the precipitate obtained at 900–1200g for 60 min. was resuspended in 2·0 ml. of PBS to form what was termed 'aggregated antigen (a)'. An equal volume of the same pool of allantoic fluid was concentrated at 1200–1400g for 2 hr. in the thin-layer rotor to give a final concentrate diluted to 2 ml., forming the 'monomeric antigen (b)'. Both antigen preparations were held overnight at 4° C. with 0·05% formaldehyde. The absence of infectious virus was confirmed, as in all experiments, by failure to induce haemagglutinin formation in embryonated hens' eggs. Each antigen was inoculated into six guinea-pigs, the animals receiving each a single intramuscular injection of 0·1 ml. of the antigen.

E. *Antigenicity of X 31 recombinant strain of influenza virus precipitated by* P.E.G. *from partly purified suspension*

The virus, partly purified and concentrated by clarification, dialyses against five volumes of buffer overnight at 4° C., pervaporation in cellophane to one-fifth volume and concentration in the thin-layer rotor with a wash cycle as described, was divided into three equal portions.

(a) The first portion was diluted eightfold with PBS and precipitated with 4% P.E.G. added as a powder, centrifuged from the supernatant (900–1200g for 60 min.), resuspended in PBS and centrifuged as before; the final deposit was resuspended in PBS to the original starting volume of this portion. After 0·1% formaldehyde inactivation this formed aggregated antigen (a).

(b) The second portion received no treatment other than an inactivation with 0·1% formaldehyde. This formed the monomeric antigen (b).

(c) The third was disrupted by shaking with ether for 5 hr. at 4° C. after Brandon, Timm, Quim & McLean (1967). After removing the ether, restoring the volume and treating with 0·1% formaldehyde the dissociated antigen (c) was formed.

In this experiment each guinea-pig was inoculated with 1·0 ml. of a 1/2 dilution of antigen. It will be seen that all experimental animals received equal amounts of virus antigen as calculated on the basis of equivalent dilutions of the common starting material (assuming no losses).

RESULTS

In Fig. 1 the remaining haemagglutinin activity of the supernatant is related to the P.E.G. concentration on material stored at 4° C. for 20 hr. The two curves represent respectively the results achieved by centrifuging at 650g for 20 min. and
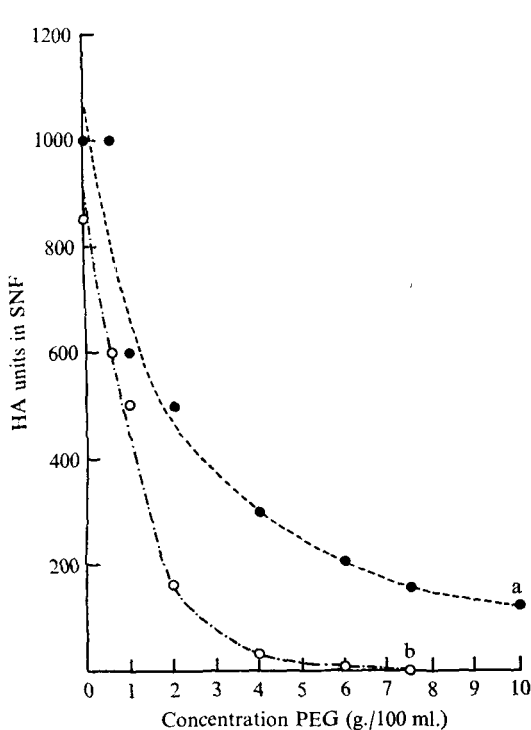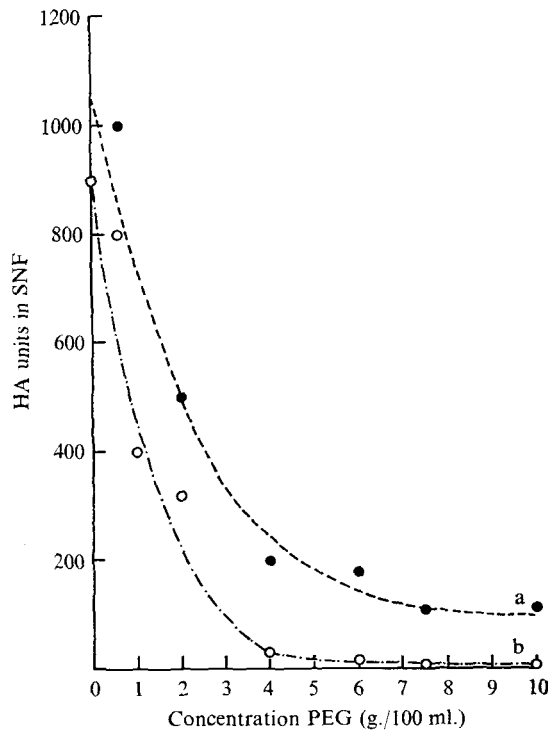
Fig. 1. Haemagglutinating activity (50 % end-points) remaining in the supernatant after centrifugation of the precipitate formed on addition of P.E.G. to different concentrations. The infected allantoic fluid was stored for 20 hr. at 4° C. prior to precipitation. (*a*) After centrifugation at 500*g* for 20 min. (*b*) After centrifugation at 1500*g* for 20 min.

Fig. 2. Haemagglutinating activity (50 % end-points) remaining in the supernatant fluid after centrifugation of the precipitate formed on addition of P.E.G. to different concentrations. The infected allantoic fluid was stored for 20 hr. at 37° C. prior to precipitation. (*a*) After centrifugation at 500*g* for 20 min. (*b*) After centrifugation at 1500*g* for 20 min.

at 1200*g* for the same time, as obtained in Expt. A. In Fig. 2 is presented similar 'precipitation' curves as in Fig. 1 but here obtained with infected allantoic fluid stored at 37° C. for 20 hr. The Hong Kong A 2/8/68 and X 31 strains behaved alike in P.E.G. precipitation. The results on the recovery of virus in terms of H.A. activity are presented in Table 1. The data are from two independent experiments.

By interpolation in Figs. 1 and 2 it is clear that approximately 90 % of the H.A. activity is removable from the SNF by 2 % P.E.G. but the H.A. (titre increase)/ (concentration factor) ratio is appreciably lower, indicating that the majority of virus particles are in a state in which they cannot participate individually in the H.A. reaction. This would be the case if they are forced into clusters by the P.E.G.

By initial treatment, as in experiment B, with 0·6 % P.E.G. followed by centrifugation, much extraneous material, some viral aggregates (possibly previously present) and mycoplasma-like particles (which were sometimes present in the egg

Table 1. *Recovery of virus following precipitation with* 2 % P.E.G.

(Precipitates spun out at 500*g* for 20 min., resuspended and precipitated from decreasing volumes of medium using 2 % P.E.G.)

Expt.	Volume (ml.)	H.A. titre	Concentration factor	H.A. titre increase
1	200	640	—	—
	10	5,120	× 20	× 8
	2·5	10,240	× 80	× 16
2	100	1,280		
	20	2,560	× 5	× 2
	2	20,480	× 50	× 16

stock (Plate 1, fig. 1) were removed. The loss of haemagglutinating activity in the supernatant resulting from such treatment was 10–15 %. Separation of filamentous from round particles was suggestive but requires further investigation.

Electron microscopy yielded informative results. Plate 1, fig. 2 is an electron micrograph of Hong Kong A 2/8/68 after concentration and purification in the thin-layer rotor. This preparation upon analytical ultracentrifugation in the Model E showed a sedimenting boundary which broadened rapidly but from which an average sedimentation coefficient of 690 Svedberg units could be calculated. The electron micrograph (Plate 2, fig. 3) was of the same pool of virus-infected allantoic fluid but precipitated with 4 % P.E.G. The intimate interlocking and binding of the virions forming large aggregates which did not disperse to the monomeric state was confirmed by centrifugation in the Model E. This precipitate obtained with 4 % P.E.G. was centrifuged under the same conditions as the virus in its monomeric state but it failed to show any sedimenting boundary as the material had already sedimented out when the required rotor velocity had been reached. Electron micrographs of the X 31 strain of influenza virus concentrated by thin-layer centrifugation and by precipitation with 4 % P.E.G. are depicted in Plate 2, fig. 4 and Plate 3, fig. 5 respectively.

The results of the antigenicity studies D and E are presented in Tables 2 and 3 respectively.

As there was some variation in the response of the individual animals in the different groups, the haemagglutination-inhibiting activity is presented as a mean value. The occasional serum from control non-injected animals similarly bled showed slight haemagglutination inhibition at 1/10 dilution but no higher. This was not considered significant.

Clearly the 'aggregated' antigen in Expt. D produced a better response than the 'monomeric' antigen.

It must be remembered that in Expt. D an 'aggregated' antigen had been subjected to a series of further procedures as compared with the control 'monomeric' antigen but that for the purpose of administering equivalent masses of antigenic material losses during such procedures were ignored. The 'aggregated' antigen was thus handicapped from the start. However, it still produced at least

Table 2. *Experiment D: immunization of guinea-pigs with Hong Kong A 2/8/68 inactivated with formaldehyde*

| | HA titre after concen- tration | Guinea-pig serum HAI titres† | | |
| | | Before immuni- zation | 3 weeks after immuni- zation | 7 weeks after immuni- zation |
Antigen*				
(a) Aggregated	3,200	< 10	37·0	13·3
(b) Monomeric	12,000–25,000	< 10	12·5	6·6

* Thirty ml. infected allantoic fluid, HA titre 2560. (a) Precipitated with 4 % polyethylene glycol, resuspended in 2 ml. buffer. (b) Concentrated to 2 ml. in the thin layer rotor.

† Six guinea-pigs inoculated with each antigen. Titres are geometric means.

Table 3. *Experiment E: immunization of guinea-pigs with X 31 recombinant strain inactivated with formaldehyde*

| | HA titre after concentration and/or dissociation | Guinea-pig serum HAI titres† | | |
| | | Before immuni- zation | 3 weeks after immuni- zation | 7 weeks after immuni- zation |
Antigen*				
(a) Aggregated	400	< 10	50	27
(b) Monomeric	1600	< 10	40	30
(c) Ether dissociated	800–1600	< 10	18‡	10

* 400 ml. infected allantoic fluid concentrated and purified by procedures described in section E (text) and divided into three portions. Portion (a) was aggregated with 4 % polyethylene glycol, portion (b) was used in the monomeric state, and portion (c) was dissociated with ether.

† Six guinea-pigs inoculated with each antigen. Titres are geometric means.

‡ Three of the six guinea-pigs died, the values after 3 weeks and 7 weeks therefore not statistically significant.

as good a response as, if not better than, the control. Furthermore, the 'aggregated' antigen had but one quarter of the haemagglutinin titre of the control.

The poor response to ether-treated virus antigen was disappointing in the light of the generally rather favourable antibody responses reported in the literature.

The authors feel that the ether treatment may have been far too harsh, fragmenting the material excessively and the significance of the result is questionable in view of the small number of animals which survived the test.

Removal of traces of P.E.G. *from the purified virus*

In view of the importance of the removal of traces of P.E.G. and extraneous egg proteins from the purified virus vaccines, the precipitate or aggregates formed by the displacement action of P.E.G. must be considered. When an influenza virus suspension is treated with the polymer the ensuing precipitate is composed of aggregates of virus particles ranging from one particle to several hundred. The

particle size distribution of the individual aggregates also depends upon the initial concentration of the virus. Thus when a suspension which had a small number of monomeric particles per unit volume is precipitated, the aggregates formed are composed of small numbers of virions. When the concentration of the infectious agent is high, the precipitate with P.E.G. is composed mainly of clusters of large numbers of monomeric units. If we regard the individual aggregates as approximately spherical in shape an important conclusion may be drawn regarding their solubility. Thus thermodynamic theory predicts that the escaping tendency or solubility from curved surfaces is inversely proportional to the radius of the curved body.

If we regard the solubility as being directly proportional to the escaping tendency or difference in molar free energy ΔF the quantitative relationship

$$\Delta F = \frac{2\gamma V}{r}$$

(see Lewis & Randal, 1923) would explain the behaviour of the clusters of particles in suspension. In this equation γ is the surface tension, V the molar volume and r the radius of the curved surface. The entities γ and V are the same for all the aggregates, therefore the radius r is the only factor which would influence the solubility of the precipitate. If a precipitate is composed of clumps of different radii it may be expected that the smaller particles will dissolve and be re-aggregated into the larger units. This transfer of monomeric particles from the small to the larger aggregates must be time-dependent and the final state of equilibrium may only be reached after a period of an hour or longer.

This appears to be the case as it is necessary, when doing precipitation of influenza virus with P.E.G., to leave the precipitated virus for a period of 1–2 hr. before further treatment. During this period the precipitated suspension, being transparent initially, becomes opaque, indicating the formation of larger aggregates. It would therefore appear that there is a very complex equilibrium condition existing amongst the various size aggregates in the suspension of the virus precipitate. It may thus be expected that an increase in the monomeric form in the suspension would favour the production of coarse precipitates which may be removed from suspension by centrifugation at low rotor velocities. Similarly by decreasing the number of monomeric forms of the virus or by performing the precipitation on infected fluid of low H.A. titre, fine precipitates will form. These will not change noticeably in degree of dispersion with time and would require higher rotor velocities for their removal from suspension.

As the degree of dispersion is dependent upon the amount of virus initially present and as the solubility of the clumps is an inverse function of their size, these facts must be considered when traces of P.E.G. are removed from a suspension. The clumps of virus have a limited solubility and the contaminating P.E.G. is completely soluble and by suspending the virus precipitate in buffer of a volume three times that of the precipitated virus suspension very little virus in the monomeric state is left in the SNF after centrifugation. By repeating the 'washing' three times the P.E.G. content may be reduced to levels at which the polymer is no longer detect-

able but retaining most of the virus in an aggregated state, indicating a concentration of less than 0·01% P.E.G. which can be detected by Nessler's reagent (Polson *et al.* 1964).

It is our experience that when the suspension is diluted in the ratio of 1 part concentrated precipitate to 99 parts of diluent the virus precipitate dissolves slowly. Washing the precipitate with large volumes of buffer is therefore not recommended.

In addition to the differential removal of P.E.G. by washing the method also ensures the removal of extraneous egg protein which co-precipitated with the influenza virus particles.

If complete recovery of the virus antigen is desired by the differential removal of the contaminating P.E.G., the suspension of the precipitate may be cooled to approximately 0° C. and then mixed with 30% ethanol in saline at −20° C. and centrifuged. Under these conditions the virus precipitate is completely insoluble while the traces of contaminating P.E.G. dissolve in the ethanol-saline. This method is termed the cryoethanol procedure. A similar method for the removal of P.E.G. from protein fractions has been described by Polson *et al.* (1964).

Electron micrographs of the virus precipitates after an additional cryoethanol treatment indicate that approximately 10–15% of the virus particles had apparently lost their nucleo-proteins. It would appear that the monomeric and filamentous forms of the virus were more resistant to ethanol than the large or giant forms which seemed to have lost their nucleoproteins, while the doughnut and filamentous forms appeared to be intact as shown in the electron micrograph (Plate 3, fig. 6).

DISCUSSION AND CONCLUSIONS

Influenza virus may be precipitated with polyethylene glycol at polymer concentrations appreciably lower than those used by Kanarek & Tribe (1967). The precipitation curves after storage at 4° and 37° C. for 20 hr. are essentially similar and it is clear that 90% or more of the haemagglutinating material, probably intact virus or, less likely, free haemagglutinin, was precipitated by P.E.G. at polymer concentrations between 2% and 4%. The shape of the curves indicate that the material being precipitated was of an inhomogeneous nature. Both the Hong Kong A 2/8/68 and the X 31 strains behaved identically in their precipitation with P.E.G. The precipitates which formed were fine aggregates of virus particles ranging from a few, i.e. 10, to more than 100 per cluster as revealed by electron microscopy. The smaller aggregates required higher centrifugal forces to sediment them from suspension than coarse protein precipitates ordinarily produced by P.E.G. These forces (1500–1900g) were nevertheless appreciably lower than those required for sedimenting the monomeric form of the virus into a pellet.

All attempts to disperse the P.E.G. virus aggregates into monomeric forms without severe damage to the virions failed. It was therefore possible to wash the precipitated virus with small volumes of suitable buffer and thus free it of traces of P.E.G. and extraneous proteins with the loss of only a small amount of virus. The haemagglutinating activity of the resuspended precipitate was one-quarter to one-

fifth of the original untreated allantoic fluid and the supernatant fluids had activities which were of the order of 1 % to 5 % of the original. This indicated that the influenza virus either had been damaged, at least in respect of its haemagglutinin, or had associated into aggregates. As P.E.G. is a relatively inert material which precipitates substances from solution by a replacement mechanism as discussed by Juckes (1971) it was concluded that the individual virus particles were not significantly altered but that they were clumped together, a conclusion supported by their appearance in the electron microscope and their behaviour in the analytical ultracentrifuge.

On comparing the electron micrographs of the virus aggregated by P.E.G. with those of the virus in the monomeric state it would appear that the haemagglutinin subunits of adjacent virions are more closely interlocked in the P.E.G. aggregated material than in material concentrated in the thin-layer rotor. This interlocking, induced by the P.E.G., may be responsible for the observed failure of such aggregates to disperse. It is of interest to note that Kanarek & Tribe (1967) found complete quantitative recovery of infectivity and an apparent increase in haemagglutinin activity in some cases after treatment of dilute myxovirus suspensions with P.E.G. This phenomenon they attributed to disaggregation of agglutinated virus particles. Complete dispersion of the aggregates is thus implied. This is in contrast with the work described in the present paper on closely related viruses but at considerably higher concentrations.

Immunization experiments with the virus in various states of aggregation indicate that the virus when aggregated is more immunogenic than the virus in the monomeric state. It is suggested that the relative stability and indispersibility of the aggregates may account for the enhanced antigenicity as assessed by the production of haemagglutination-inhibiting antibody in guinea-pigs. In the present context it is clear that the haemagglutinating activity tests are not applicable in assessing antigenic content or antigenicity of an aggregated antigen preparation.

Very recently Larin & Gallimore (1971) have shown that the relationship between haemagglutinating activity and immunogenicity of an influenza antigen is merely coincidental; this inference is supported in the present work.

The results suggest that P.E.G. is of value in viral vaccine production. Coarse debris including mycoplasmas may be removed by low-speed centrifugation in the presence of very low concentrations of P.E.G. An increase in P.E.G. concentration may cause aggregation of virus particles, such aggregates being easily concentrated and washed free of contaminants by low-speed centrifugation, and such aggregates constitute good antigenic material. Their inactivation poses no problem since the virus may be inactivated before precipitation with P.E.G.

It is suggested that the P.E.G. precipitation followed by washing the precipitate with buffer to free it of extraneous protein and traces of P.E.G. be used as an alternative method to the very costly technique of zonal centrifugation of Gerin & Anderson (1969) for the purification of influenza and other viruses in the production of vaccines for human and animal immunization.
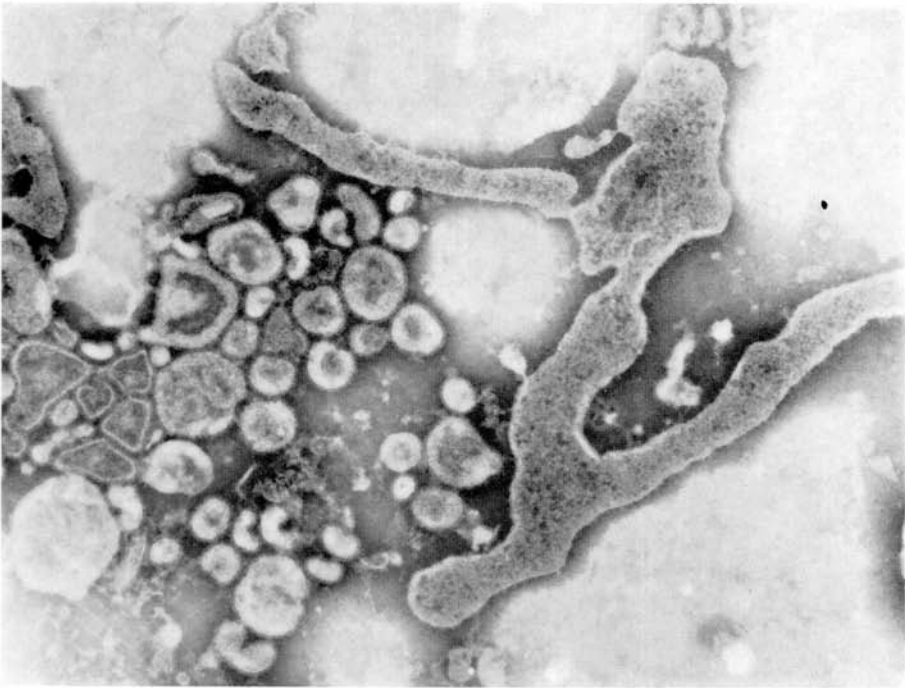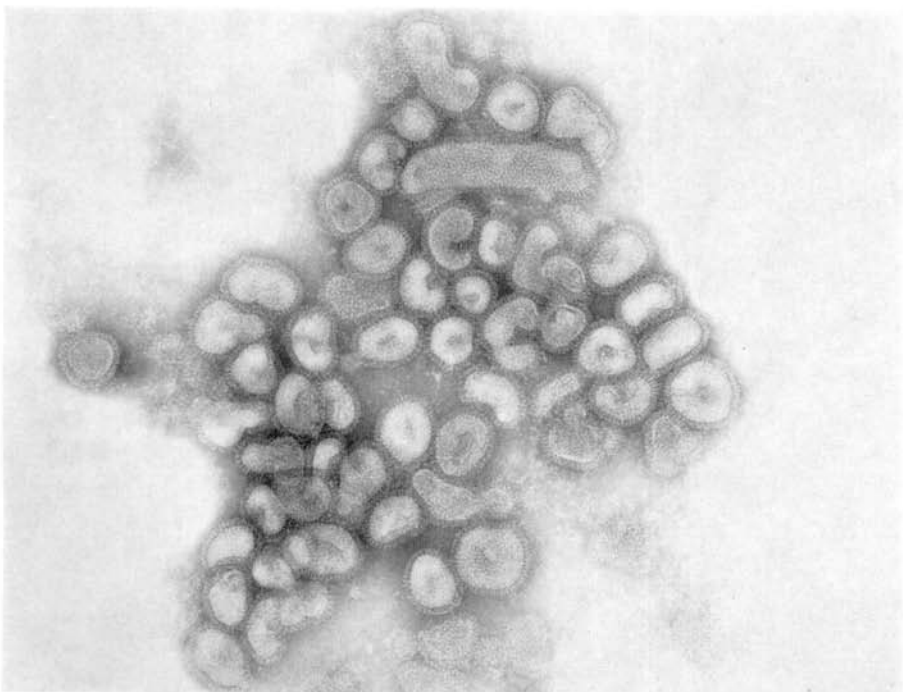
Fig. 1

Fig. 2

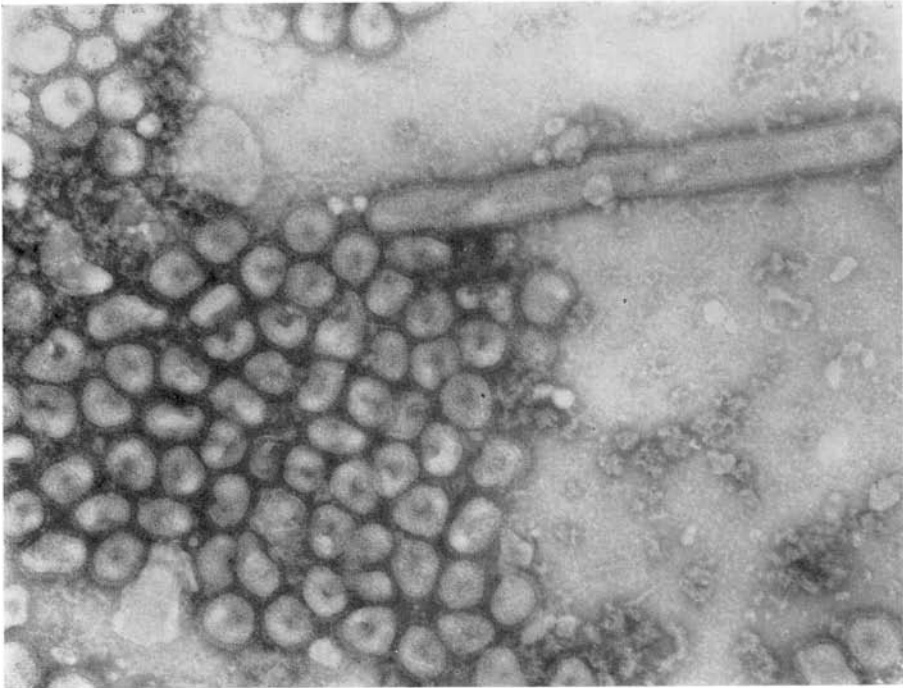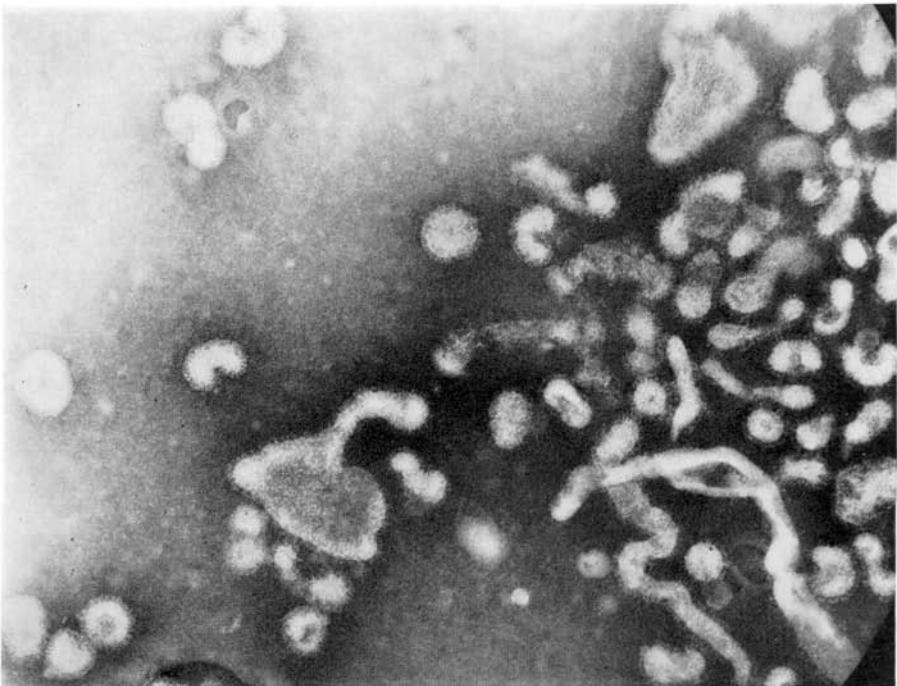A. POLSON, A. KEEN, C. SINCLAIR-SMITH AND I. G. S. FURMINGER   (*Facing p.* 264)

Fig. 3

Fig. 4

A. POLSON, A. KEEN, C. SINCLAIR-SMITH AND I. G. S. FURMINGER
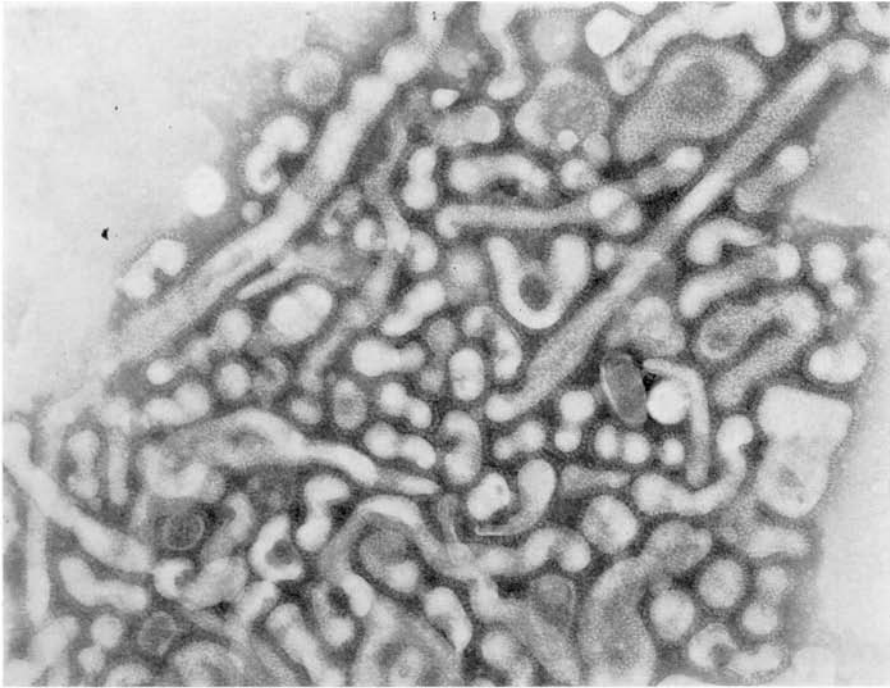
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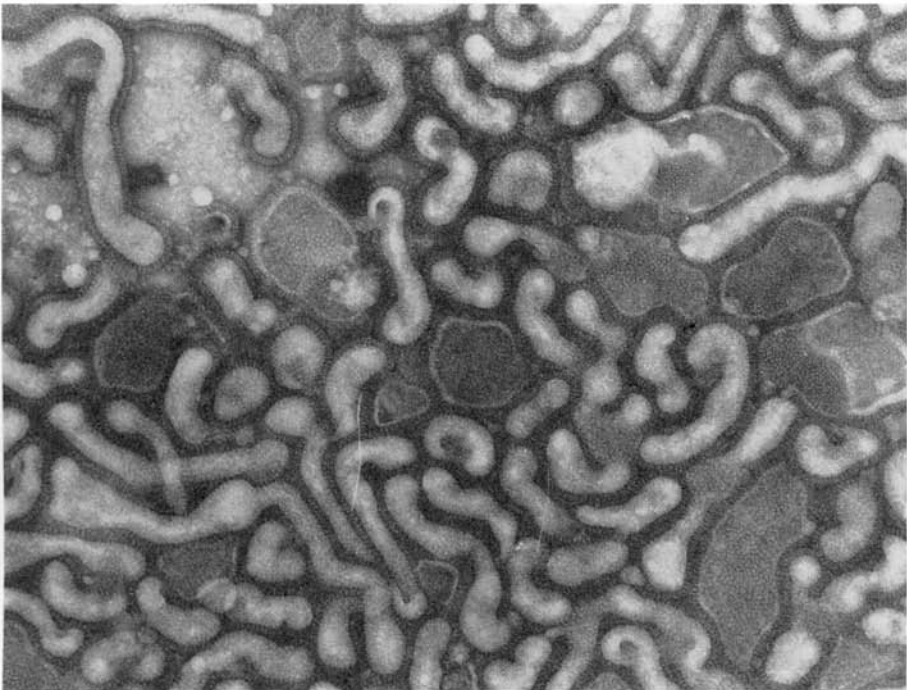
Fig. 6

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Electron micrograph of fraction obtained on precipitation of infected allantoic fluid with 0·6 % P.E.G. Note the large mycoplasm-like particle. × 60,000.

Fig. 2. Electron micrograph of Hong Kong A2/8/68 concentrated with the thin-layer rotor at 500g for 60 min. Note the loose association of the virus particles. (Compare Fig. 3.) × 60,000.

PLATE 2

Fig. 3. Electron micrograph of Hong Kong A2/8/68 influenza virus precipitated with 4 % P.E.G. Note the close association of the virus particles. (Compare Fig. 4.) × 60,000.

Fig. 4. Electron micrograph of strain X31 influenza virus concentrated by thin-layer ultracentrifugation at 1500g for 60 min. Note the loose association of the virus particles. (Compare Fig. 5.) × 60,000.

PLATE 3

Fig. 5. Electron micrograph of strain X31 influenza virus concentrated by precipitation with 4 % P.E.G. Note the close association of the virus particles. × 60,000.

Fig. 6. Electron micrograph of influenza X31 initially precipitated with 4 % P.E.G. then resuspended in 4 volumes of buffer and finally reprecipitated in 30 % ethanol at − 20° C. Note that the smaller virus particles are intact and that the larger particles seem to be devoid of internal components. × 60,000.