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A surface antigen influenza vaccine. 1. Purification of haemagglutinin and neuraminidase proteins

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SUMMARY

Influenza virus was centrifuged in a KII rotor through a sucrose gradient containing Triton N101, a non-ionic surfactant. The micelles of surfactant formed a band in the gradient. As virus particles passed through the surfactant, the haemagglutinin and neuraminidase proteins were stripped from the surface and remained near the surfactant micelles. The residual virus particles sedimented into a denser region of the gradient and were thus separated from the haemagglutinin and neuraminidase antigens. Fractions containing the surface antigens were pooled and Triton was removed by phase-separation at the cloud point.

INTRODUCTION

Influenza vaccines have been produced for many years. With the earlier vaccines the main problem was that of unwanted side reactions produced by contaminating egg protein. These reactions have been minimized by using highly purified virus that has been separated from contaminants by density gradient zonal centrifugation. For many years it had been realized that viruses could be purified by density gradient centrifugation but it was not until the development of the continuous flow KII zonal centrifuge (Gerin & Anderson, 1969) that the process was used commercially. Reimer *et al.* (1967) showed that it was possible with these rotors to separate the virus from the host protein. Vaccines containing virus purified by zonal centrifugation were less reactive than vaccines prepared from virus that had been sedimented as a pellet and resuspended (Peck, 1968). These zonally purified vaccines were of low pyrogenicity but the pyrogenicity could never be completely eliminated.

However, Webster & Laver (1966) showed that pyrogenicity was reduced by disruption of the influenza virus particle with sodium dodecyl sulphate or sodium deoxycholate. Similar results were also obtained by disruption of virus with diethyl ether (Cromwell, Brandon, McLean & Sadusk, 1969). These vaccines were disrupted preparations of whole virus which, in most cases, had not been purified by zonal centrifugation before disruption and were not subsequently purified. Attempts have been made to purify the virus before and after splitting. Neurath, Rubin, Sillaman & Tint (1971) adsorbed virus from allantoic fluid into calcium phosphate columns and eluted subunits with tri-(*N*-butyl) phosphate while Davenport *et al.* (1964) disrupted virus with a Tween-ether mixture and precipitated

the ribonucleoprotein with lanthanum acetate. Neither of these approaches produced a product that contained only haemagglutinin and neuraminidase.

It is these two surface proteins which induce protective antibody. Hobson, Curry, Beare & Ward-Gardner (1972) showed that protection from influenza infection was proportional to the serum haemagglutination-inhibition (HAI) titre. However, antibody to neuraminidase has also been shown to confer immunity (Schulman, Khakpour & Kilbourne, 1968; Monto & Kendal, 1973; Couch *et al.* 1974), and for maximum protection antibodies to both surface proteins are probably required (Slepushkin *et al.* 1971).

It was shown by Hoyle, Horne & Waterson (1961) that a diethyl ether/Tween mixture broke up the lipid membrane of the influenza virion liberating the internal component. The broken lipid membrane with neuraminidase and haemagglutinin attached rounds up into little rosettes. Other organic solvents such as tri-(*N*-butyl) phosphate may be expected to act in a similar way. Detergents such as sodium deoxycholate have been shown to remove haemagglutinin and neuraminidase from the lipid membrane, leaving a subviral particle devoid of surface projections (Skehel, 1971).

Proteolytic enzymes have also been used to remove surface proteins from the virus. Brand & Skehel (1972) showed that under specified conditions bromelain would liberate haemagglutinin from virus particles and destroy neuraminidase. The liberated haemagglutinin was purified and crystallized.

Corbel, Rondle & Bird (1970) showed that Triton N101, a non-ionic detergent, stripped the neuraminidase and haemagglutinin from the lipid membrane leaving the core of the virus intact. The antigenicity of both haemagglutinin and neuraminidase was maintained. This is the type of detergent required for the preparation of a purified haemagglutinin and neuraminidase vaccine as these proteins can be stripped from the virus and separated easily from the viral cores by density gradient centrifugation.

In the following report we describe a technique for the commercial production of a vaccine consisting only of the surface proteins of the virus, the haemagglutinin and neuraminidase. These proteins were split from the virus by Triton N101, purified and adsorbed to aluminium hydroxide to enhance antigenicity.

MATERIALS AND METHODS

Virus strains

X-31 was a recombinant virus (Kilbourne *et al.* 1971) which contained the haemagglutinin and neuraminidase of A3/Aichi/2/68 but the egg growth characteristics of A0/PR8/34.

A/England/42/72 and B/Victoria/98926/70 were obtained from the W.H.O. Laboratories, Mill Hill, London.

Virus cultivation

Influenza virus ($10^{4.0}$ EID 50) of each of the above strains was inoculated by the allantoic route into 11-day-old embryonated hens' eggs. They were chilled at 4° C.

and the allantoic fluid was harvested. β-propiolactone was added to a final concentration of 0·1 % to inactivate the virus.

Purification of virus

Throughout the work the KII zonal centrifuge (Electro-Nucleonics Inc.) was used at room temperature. Allantoic fluid (140 l.) was clarified at low speed (15,000 ***g***) in an Alfa-Laval centrifuge at a rate of 2 l./min. to remove gross contaminants, e.g. erythrocytes. The virus was removed from the allantoic fluid by continuous flow centrifugation through a sucrose density gradient. The gradient was established in the KII rotor by adding sucrose (60 %, w/w, 1·4 l.) to the stationary rotor and slowly adding phosphate buffered saline (PBS) (1·8 l.) as a top layer.

The vertical gradient was reorientated into the horizontal axis by centrifugation at 3000 rev./min. for 15 min. The rotor was accelerated and a fast flow of PBS (20–30 l./hr.) was started. When the rotor reached a speed of 35,000 rev./min. (90,000 ***g***) the PBS was replaced with clarified allantoic fluid at a flow rate of 30 l./hr. Under these conditions virus particles entered the sucrose gradient and formed a band at their isopycnic density. When all the virus had been centrifuged across the gradient, the rotor was operated at 90,000 ***g*** for a further hour to effect better separation of the virus.

At the end of centrifugation, the rotor was halted and the gradient reorientated in the vertical axis. Fractions (100 ml.) were collected from the bottom of the rotor and those containing a high concentration of virus, as indicated by haemagglutination, were pooled and diluted to 5 % sucrose with PBS. The final volume was approximately 10 l.

Disruption of virus

The virus was disrupted by centrifuging influenza virus through a sucrose gradient containing Triton N101, in a KII rotor.

A gradient was established by adding PBS (0·01 M, pH 7·6) (1·8 l.) containing Triton N101 (1 %, v/v) to sucrose (60 %, w/w, in PBS) (1·4 l.) also containing Triton N101 (1 %, v/v). The gradient reorientated as the rotor accelerated at 90,000 ***g*** and the Triton micelles formed a band within minutes.

Purified virus diluted to 10 l. as described above was allowed to flow across this gradient and disruption occurred as the virus particles sedimented through the band of Triton. The rotor was operated for a further hour at 90,000 ***g*** to separate the split products. Fractions (100 ml.) were collected from the bottom of the rotor.

Removal of Triton

The fractions from the gradient which contained surface proteins as indicated by the peak of neuraminidase activity were pooled and phosphate buffer (2 M, pH 7·0) was added to give a final concentration of 0·5 M. Under these conditions, the cloud-point of the surfactant was exceeded at room temperature. The solution was allowed to stand at room temperature overnight and the Triton separated as a narrow upper layer. The lower phase containing the viral subunits was removed.

Electron-microscopy

Dilute samples were concentrated by sedimentation and resuspended in PBS. Samples were added to carbon-coated formvar grids and salts were removed by addition and removal of distilled water to the grid. Phosphotungstic acid (2·5 %, w/v) pH 6·8 was used as a negative stain for the samples.

Neuraminidase assay

The method of Warren (1959) was used to assay *N*-acetyl neuraminidase acid (NANA) released by the action of neuraminidase on fetuin. The incubation mixture consisted of 0·05 ml. fetuin (24 mg./ml.), 0·05 ml. phosphate buffer (0·4 M, pH 5·9), 0·05 ml. saline (0·85 %, w/v) and 0·05 ml. neuraminidase solution. The latter was diluted so that after 4 hr. incubation at 37° C., the NANA released gave a colour reaction with an absorbance between 0·2 and 0·8 at 550 nm. The results were either reported as absorbance or were converted by comparison with a standard NANA graph to μmol NANA released per hr. per ml. of solution.

Phospholipid assay

Lipid was extracted from virus according to the method of Bligh & Dyer (1959), using a final ratio of sample:methanol:chloroform of 0·8:2:1. The lipid was extracted finally into chloroform. This was evaporated to dryness in a water-bath and the lipid phosphorus was oxidized to inorganic phosphate by perchloric acid. Inorganic phosphorus was then assayed by formation of a blue phosphomolybdate complex based on the method of Allen (1940). The method was modified slightly to increase the sensitivity. After evaporation of chloroform, perchloric acid (1 ml.) was added to the combustion tube which was carefully heated until the solution was clear. In the samples which contained a large amount of carbon the digestion time was approximately 30 min. and additional perchloric acid was required. For the samples containing less carbon, the perchloric digest became clear after about 7 min. and only 0·6 ml. of perchloric acid was added initially. The final volume of perchloric acid after digestion was 0·4 ml. Distilled water (1 ml.) was added to each sample followed by amidol reagent (1 %, w/v, 2·4 diaminophenol HCl in 20 %, w/v, sodium metabisulphite) (0·4 ml.) and ammonium molybdate solution (8·3 %, w/v) (0·2 ml.). A blue colour developed and was read at 610 nm. within 5–30 min. of molybdate being added. A set of standard phosphate solutions was always assayed in parallel with unknown samples. A linear response was obtained for solutions of phosphate containing between 0–14 μg. phosphorus/ml. The upper absorbance reading at 610 nm. was 0·7.

Protein assay

Protein was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951), modified for the Autoanalyser. Bovine plasma albumin was used as a standard. Interference by Triton was eliminated by increasing the reaction temperature to 95° C.

Sucrose assay

Sucrose was determined in fractions from a gradient by refractivity measurements at 20° C., on an Abbe '60' refractometer.

Triton N101 assay

Triton N101 has a characteristic absorption spectrum in the ultra-violet with a peak at 277 nm. Solutions of Triton were prepared in PBS at concentrations from 0·01 %, v/v, to 0·07 %, v/v. A standard graph of absorbance at 277 nm. against concentration, was constructed. The relation was linear and the Triton concentration in samples was estimated by comparison.

Polyacrylamide gel electrophoresis

Samples containing 1–2 mg. protein/ml. in PBS were disrupted in a mixture of 5 M urea, 1 %, w/v, SDS and 2 %, v/v, *B*-mercaptoethanol by heating at 100° C. for 1 min. The mixture was cooled before addition to the polyacrylamide gel.

The polyacrylamide gels were prepared in glass tubes of 5 mm. internal diameter and contained 4·5 %, w/v, acrylamide and 0·13 %, w/v, bisacrylamide. The gel buffer contained phosphate (0·05 M, pH 7·2), EDTA (0·01 M), SDS (0·1 %, w/v), urea (5 M) and tetramethyl-ethylenediamine (0·1 %, v/v). The polymerization was catalysed with 0·1 % ammonium persulphate.

Electrophoresis was carried out at 3 *V.*/cm. for 4 hr. in phosphate buffer (0·05 M, pH 7·2), containing 0·01 M EDTA and 0·1 %, w/v, SDS. The volume of sample added to the gel never exceeded 0·2 ml. Bromophenol blue was added to allow the course of electrophoresis to be followed. When the bromophenol blue band had migrated 5 cm., the gels were removed and soaked in trichloracetic acid (12 %, w/v). White bands of precipitated protein appeared after about 1 hr. Permanent records were made by scanning the gels, without staining, in a densitometer (Joyce-Loeble 'Chromascan'), with a 405 nm. filter.

The gels were stained by the periodic acid-Schiff's technique to detect glycoproteins. They were soaked for 1 hr. in periodic acid (1 %, w/v, in 3 %, v/v, acetic acid) and the oxidation products were leached out by immersing the gels for 24 hr. in distilled water. They were then placed for 1 hr. in acid fuchsin solution in a sealed tube and finally stored in sodium metabisulphite solution (1 %, w/v). The glycoproteins were stained magenta by this method and were scanned at 550 nm. in the densitometer.

Identification of viral polypeptides on polyacrylamide gels

X-31 virus was disrupted with SDS and the proteins were separated by electrophoresis on cellulose acetate membranes (Schild & Pereira, 1969). The membranes were cut into segments and the proteins eluted. Haemagglutinin and neuraminidase were identified by their activities. To confirm the location of these two proteins and identify ribonucleoprotein and matrix protein, narrow longitudinal strips (2 mm. wide) were removed from a membrane and placed on a glass slide covered with agar. Strips of filter paper soaked in monospecific antiserum were placed

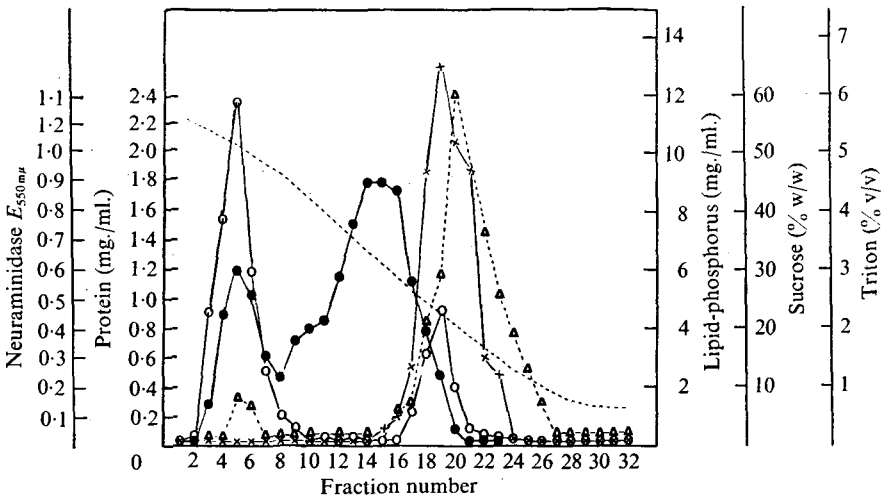

Fig. 1. Purification of X-31 haemagglutinin and neuraminidase proteins on a sucrose gradient containing Triton N101. A dilute suspension (10 l.) of intact virus (3,200 i.u./ml.) was centrifuged under continuous flow conditions at 10 l./h. and 90,000 *g* through a KII zonal rotor containing Triton N101 (1%, v/v) incorporated into a 0–60%, w/w, sucrose gradient in PBS. The centrifuge was operated for a further hour, after addition of virus and was then halted. The gradient reorientated and fractions (100 ml.) were collected and assayed for protein ○—○, neuraminidase ●—●, Triton N101 ×—×, phospholipid △—△ and sucrose - - - - -.

alongside the electrophoresis membrane. The slides were kept in a humidity cabinet overnight and precipitin lines developed in the agar. The monospecific antiserum was kindly provided by Dr G. C. Schild (M. R. C., Hampstead, London) and to identify the X-31 proteins we used rabbit antiserum to Hong Kong haemagglutinin, A2 neuraminidase, A2 ribonucleo-protein and A2 matrix protein.

To obtain sufficient amounts of individual proteins to act as markers on polyacrylamide gels, electrophoresis was carried out using about six cellulose acetate membranes. Proteins were eluted from the relevant sections and concentrated by ultrafiltration through an Amicon membrane (PM10).

EXPERIMENTAL

Inactivated whole virus of the strains studied was concentrated and purified from 140 l. of allantoic fluid in the KII zonal centrifuge, as described in Materials and Methods. Fractions containing whole virus were pooled and diluted. The virus was recentrifuged across a sucrose gradient containing Triton N101. The virus entered the gradient and was disrupted. The viral components separated with continued centrifugation. Fractions (100 ml.) were collected and assayed for protein, neuraminidase, phospholipid and Triton N101.

RESULTS

With X-31 influenza virus, two peaks of protein were located (Fig. 1), the larger peak being at the dense end of the sucrose gradient (fractions 3, 4, 5, 6). Electron-microscopic examination of the peak fraction (fraction 5), showed particles which

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had lost their surface projections (Plate 1*a*). There was no haemagglutinating activity associated with this peak although a small amount of neuraminidase was detected. The results of electron-microscopy were confirmed by polyacrylamide gel electrophoresis (Fig. 3*b*), which showed that fraction 5 contained only matrix protein and ribonucleoprotein. Glycoprotein was not detected in this fraction by periodic acid-Schiff stain. Neuraminidase shown by the sensitive colorimetric enzyme assay was presumably insufficient for detection by periodic acid-Schiff reagents.

Further evidence that this protein peak contained stripped particles and not whole virus was its location in the gradient. In our experience with the KII rotor, whole influenza virus particles, when centrifuged under the conditions described, formed a band in the 42%, w/w, sucrose region of the gradient. The particles which we found in fraction 5 are in 50–52%, w/w, sucrose. They are denser than intact virus possibly because they contain very little lipid. Phospholipid was found in another part of the gradient in fractions containing surfactant and was presumably solubilized within the micelles.

The other protein peak in Fig. 1 coincided with the position of the Triton micelles in fraction 19. When Triton was removed from this fraction by phase-separation at the cloud-point (Materials and Methods), phospholipid was also lost. A sample of fraction 19 was examined in the electron microscope after removal of Triton and stellate aggregates of haemagglutinin were observed (Plate 1*b*).

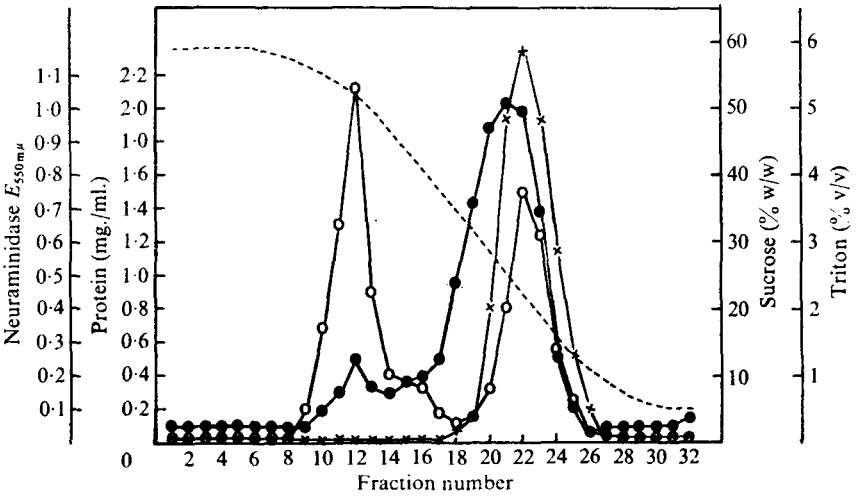
Analysis of this fraction by polyacrylamide gel electrophoresis showed that the two glycoprotein subunits of haemagglutinin, HA1 and HA2 (Fig. 3*c*) were present. The fractions containing neuraminidase activity of X-31 (Fig. 1, fractions 14 and 15) were examined, after concentration, in the electron-microscope. 'Cartwheel' formations were observed. The identity of the protein in these fractions was confirmed by polyacrylamide electrophoresis (Fig. 3*d*).

With X-31 virus, the neuraminidase subunits were always found in slightly denser sucrose than the haemagglutinin subunits. With other strains, such as A/England/42/72 and B/Victoria/98926/70, both haemagglutinin and neuraminidase activities were associated with the protein at the top of the gradient (Figs 2*a* and 2*b*) and in the electron-microscope homopolymers of both types of subunit were apparent (Plate 1*c*).

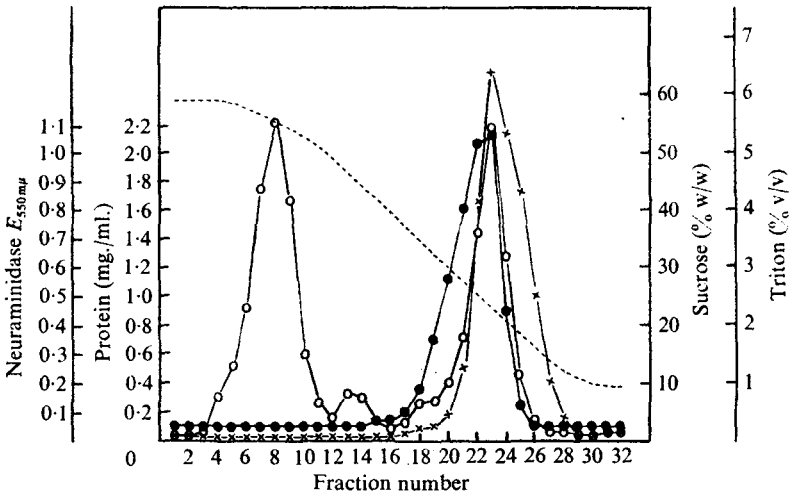
There was also a much smaller proportion of the total neuraminidase activity in the first protein band from the A/England/42/72 gradient and no neuraminidase activity in similar fractions from the B/Victoria/98926/70 gradient, indicating perhaps a more efficient disruption in these strains.

Detection of haemagglutinating activity was precluded in those fractions from the gradients which contained Triton as this is a haemolytic surfactant. The single radial diffusion technique (Schild, Henry-Aymard & Pereira, 1972) which is now used to detect haemagglutinin had not been developed when the rest of this work was carried out.

In current studies where specific anti-haemagglutinin serum is available for assay of haemagglutinin by radial-diffusion, the distribution of this protein coincides with the protein at the top of the gradient.



(*a*)



(*b*)

Fig. 2. Purification of A/England/42/72 and B/Victoria/98926/70 on a sucrose density gradient containing Triton N101. Distribution of protein ○—○, neuraminidase ●—●, Triton N101 ×—×, and sucrose ------, in fractions from a gradient containing (*a*) A/England/42/72, (*b*) B/Victoria/98926 and Triton N101. For experimental details see legend to Fig. 1.

DISCUSSION

Influenza virus was concentrated and purified from allantoic fluid on a sucrose gradient by zonal centrifugation. The virus was then centrifuged through a band of Triton micelles in a sucrose gradient. As the virus particles passed through the high concentration of surfactant, the surface proteins were removed. The stripped particles sedimented to a denser part of the gradient and thus separation of the 'core' proteins of the virion from the surface proteins was achieved.

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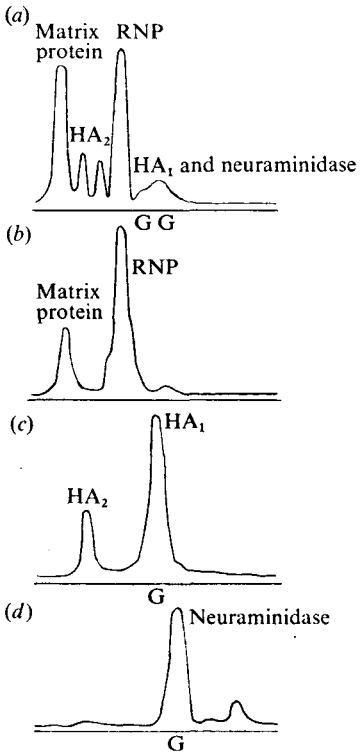

Fig. 3. Densitometer tracings of X-31 fractions from a density gradient, after electrophoresis on polyacrylamide gels. Samples containing 1–2 mg. protein/ml. were disrupted in a mixture of 5 M urea, 1 %, w/v, SDS and 2 %, v/v, B-mercaptoethanol, by heating at 100° C. for 1 min. The gels contained 4·5 %, w/v, acrylamide and 0·13 %, w/v, bisacrylamide in a phosphate buffer (0·05 M, pH 7·2) containing EDTA (0·01 M), SDS (0·1 %, w/v), urea (5 M), tetramethyl-ethylenediamine (0·1 %, v/v) and ammonium persulphate (0·1 %, w/v). Sample (100 μl.) containing bromophenol blue was added to each gel and electrophoresis was carried out at 3*V*./cm. for 4 hr. in phosphate buffer (0·05 M, pH 7·2) containing EDTA (0·01 M) and SDS (0·1 %, w/v). The gels were run simultaneously and the direction of migration was from right to left. After electrophoresis the protein bands were fixed by soaking for 24 hr. in trichloracetic acid (12 %, w/v). The bands were scanned without staining and the gels were then stained for glycoprotein (G) by the periodic acid-Schiff technique. Polypeptides were identified according to the method outlined in Materials and Methods. (*a*) X-31 whole virus; (*b*) stripped virus particles from fraction 5 (Fig. 1); (*c*) haemagglutinin from fraction 19 (Fig. 1); (*d*) neuraminidase (× 10 concentrate) fractions 14 and 15 (Fig. 1).

The latter remained as a discrete band on the gradient near to or coincidental with the Triton micelles. Phospholipids were also found in this region of the gradient, presumably solubilized within the Triton micelles.

The fractions containing the surface antigens also contained Triton and phospholipid. These contaminants were removed from the pooled fractions by addition of phosphate buffer which caused the cloud-point of the surfactant to be exceeded at room temperature.

Triton micelles separated as an upper phase and removal of surfactant and con-

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comitant removal of phospholipid was possible. This technique provided a simple means of removing both contaminants from the haemagglutinin and neuraminidase proteins. Residual Triton was of the order of 0·05%, v/v.

Electron microscopy confirmed the analytical data for the fractions from the gradient. Thus, viral particles without surface projections were found in the dense sucrose protein peak while aggregates of the surface proteins were seen in the concentrated samples from the top of the gradient.

Analysis on polyacrylamide gels showed matrix protein and ribonucleoprotein in the protein peak at the dense end of the sucrose gradient and with X-31 virus HA (1) and HA (2) were detected in the other protein peak. For this strain the neuraminidase separated from the haemagglutinin and the relevant fractions containing neuraminidase activity were concentrated by ultrafiltration through an Amicon PM10 membrane so that they could be detected on the polyacrylamide gels by periodic acid-Schiff reagent. Small degrees of impurity were also concentrated by this method (peak on right of Fig. 3*d*). The small peak (third from left) in Fig. 3*a* is also an impurity of egg origin carried through the purification procedure.

Of the strains of virus examined, only the neuraminidase of X-31 separated from the haemagglutinin. The haemagglutinin and neuraminidase activities of A/England/42/72 and B/Victoria/98926/70 coincided with each other and with the minor protein peak. This was also the case in the later experiments with A/Port Chalmers/1/73 and B/Hong Kong/8/73. For all these strains, after removal of surfactant, homopolymers of haemagglutinin and neuraminidase were observed in the electron microscope but mixed polymers were not seen.

The zonal techniques provided a means of producing purified haemagglutinin and neuraminidase on a commercial scale. It was hoped that a vaccine prepared from these surface antigens might provide a readily acceptable alternative to whole virus influenza vaccine. The problem with any disrupted vaccine, and especially with purified surface antigens of relatively small size, is retention of immunogenicity.

To overcome the reduced antigenicity of the surface antigens prepared by Triton treatment, aluminium hydroxide was added. This adsorbed preparation was then of comparable potency to whole virus vaccine when assayed in chicks (Brady & Furminger, 1976), hamsters (Jennings, Potter, McLaren & Brady, 1975), ferrets (Brady & Furminger, 1975) and man (Brady, Furminger & Stones, 1976).

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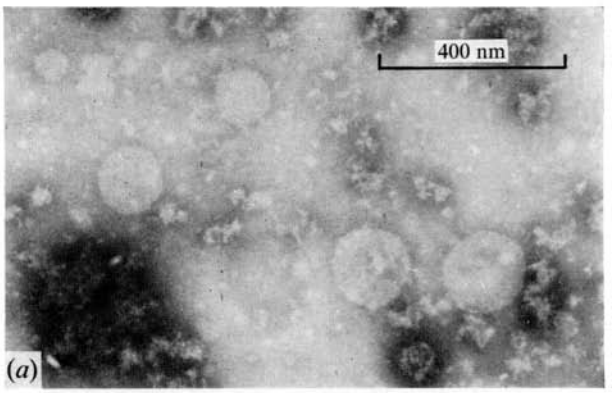
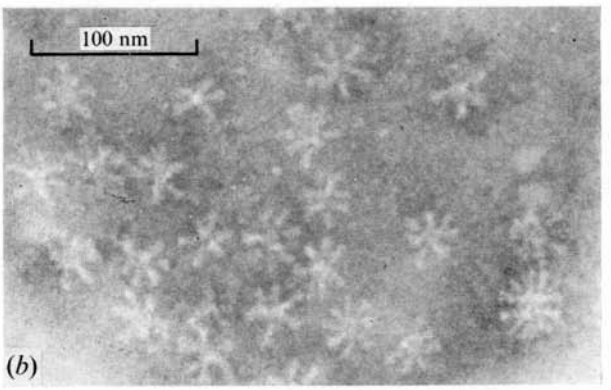
EXPLANATION OF PLATE 1

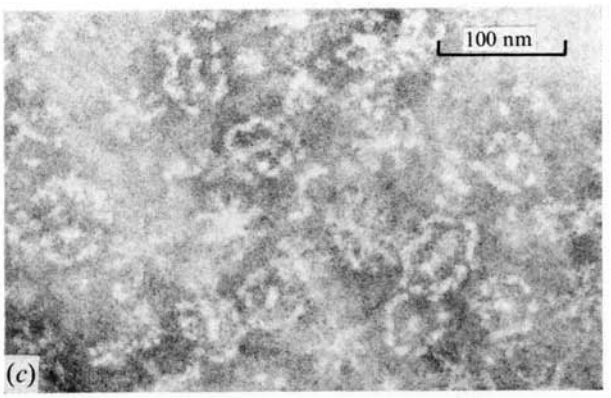
(*a*) X-31 virus particles found in fraction 5 (see figure 1) of a sucrose density gradient containing the surfactant Triton N101. The particles have been stripped of surface projections by the surfactant.

(*b*) Stellate aggregates of X-31 haemagglutinin from fraction 19 of the sucrose gradient (see figure 1). Triton N101 was removed by phase separation at the cloud-point.

(*c*) Surface antigens of A/England/42/72 from a pool of fractions 17–26 inclusive (Fig. 2*a*). After the removal of Triton N101, homopolymers of the surface proteins were apparent. The 'cartwheel' formations of neuraminidase and the stellate aggregates of haemagglutinin are shown.







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