Evaluation of Some Methods of Concentration and Purification of Influenza Virus *

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A number of techniques have been developed in the past 15 years for the concentration and purification of influenza virus, but there has been no comparative evaluation of their efficacy. In this paper the authors report on such an assessment, carried out with particular reference to the suitability of the various techniques for large-scale production of influenza vaccine, the aim being to recover a relatively pure antigen by simple means and without undue loss in the haemagglutinin and virus contents.

The purest product was obtained with two cycles of aluminium phosphate treatment. Next in order came ultracentrifugation with and without methanol precipitation, red cell adsorption, single-cycle aluminium phosphate treatment, and finally the zinc hydroxide method.

INTRODUCTION

Several methods have been described for the concentration and purification of influenza virus.

The use of adsorption on and elution from chicken red cells has been the basis of the methods proposed by Hirst (1941) and McClelland & Hare (1941). Francis & Salk (1942) used the method of adsorption on and elution from embryonic chicken red cells to achieve a tenfold concentration of the virus. Bodily et al. (1943) used alum, while Salk (1945) and Stanley (1945) used calcium phosphate for precipitation and concentration of the virus and Himmelweit (quoted by Miller & Schlesinger, 1955) used aluminium phosphate for the purpose. Stanley (1944, 1945) and Taylor et al. (1945) used the Sharples centrifuge to produce influenza vaccine on a commercial scale based on Elford & Andrewes' (1936) method of high-speed centrifugation. The successful use of ethyl or methyl alcohol for purifying proteins and important enzymes led Cox et al. (1947) to adopt methanol precipitation of influenza virus from infected allantoic fluids as the method of choice for the production of influenza vaccine on a large scale. Recently Miller & Schlesinger (1955) used adsorption of the virus on columns of aluminium phosphate gel and elution by small shifts in salt concentration and in pH to obtain highly purified influenza virus in studies on viral nucleic acids.

Thus, there are several methods which could be used for the concentration and purification of the virus. But, since Stanley's paper in 1944 comparing the four methods—(a) high-speed centrifugation, (b) adsorption on and elution from adult fowl red cells, (c) freezing and thawing, and (d) cell adsorption and elution of the virus using embryonic red cells—there has been no report on the relative merits of the newer techniques. It seemed desirable to assess the efficacy of the newer methods in order to determine which of them would be most suitable for the production of influenza vaccine on a large scale. With this object in view the methods employing (a) high-speed centrifugation, (b) red cell adsorption and elution, (c) methanol precipitation and (d) adsorption on aluminium phosphate gel and elution were studied. These were compared with a modification of the method using zinc hydroxide described by Newton & Bevis (1959) for the purification of animal viruses such as vesicular stomatitis and vesicular exanthema of swine virus.

MATERIALS AND METHODS

Virus

Five antigenically representative strains were selected for the study. They were: (a) PR8 (type A), (b) FM1 (type A1), (c) PAR (non-avid A2), (d) Gilbert (avid A2) at low (second) egg passage (LEP) and

high (12th) egg passage (HEP) levels, and (e) Crawley (type B).

Infected allantoic fluids

Ten-day-old hen's eggs were inoculated with 0.25 ml of a 10^{-3} dilution of the virus in nutrient broth. The eggs were reincubated for another 46-48 hours at 36°C and then transferred to 4°C. Clear allantoic fluids were collected aseptically, pooled, tested for sterility and used as the source of virus. Storage of the allantoic fluid at $-20°C$ and subsequent thawing generally led to the formation of precipitates, which were found to be responsible for some of the irregular results obtained in titrations of the virus. To overcome this, the infected fluids were, as a rule, clarified by centrifugation at 3000 r.p.m. for 5 minutes. However, this procedure led to a loss in the haemagglutination (HA) and virus titres, the fall being related to the period of storage in the deep-freeze and the quantity of precipitate present. When there was much precipitate after prolonged storage, the loss in the HA and virus titres was considerable.

The clear supernatant was divided into 50-ml lots and subjected to various purification procedures.

High-speed centrifugation

Infected allantoic fluid was centrifuged in a Spinco ultracentrifuge at 40 000 r.p.m. for 90 minutes at $\pm4°C$. The supernatant containing no haemagglutinin was discarded. The deposit was suspended in 0.1 M phsophate buffer (pH 7.0), the volume of buffer used being half the original volume of allantoic fluid. It was found that strains such as PR8 sedimented fully on centrifugation at 40 000 r.p.m. for 30-60 minutes, while the Asian strains did not. Therefore the centrifugation was carried out for 90 minutes, by which time practically all the virus sedimented from the allantoic fluids.

Red cell adsorption method

The infected allantoic fluid was mixed with washed and packed adult fowl cells to give a 5% red cell concentration and kept at 4°C for 60-90 minutes for adsorption of the virus to the cells. The mixture was occasionally shaken during this period, at the end of which it was centrifuged at 3000 r.p.m. for 15 minutes to pack the red cells. The packed cells were then suspended in 25 ml of 0.1 M phosphate buffer (pH 7.0) and kept in a water-bath for elution of the virus. The temperature of the bath was slowly raised to 37°C to avoid lysis of

the red cells. After 2½ hours at 37°C with occasional shaking, the mixture was centrifuged at 3000 r.p.m. for 10 minutes and the supernatant stored in neutral glass bottles. A second elution was also carried out with 10 ml of the same buffer for another 2½ hours. The second eluate was generally slightly discoloured owing to lysis of the red cells and was therefore not used.

Methanol precipitation

The method described by Cox et al. (1947) and later modified by Moyer et al. (1950) was adopted. Methyl alcohol, chilled at $-20°C$, was slowly added with a capillary pipette to infected allantoic fluids kept in an ice-bath at $-5°C$ with periodic shaking. The amount of alcohol needed for each strain for complete precipitation varied. The temperature of the alcohol-virus mixture was never allowed to rise above $-5°C$ in order to avoid denaturation of the proteins. After addition of the methanol, the mixture was shaken well and allowed to stand at $-5°C$ for approximately 3 hours and then centrifuged at 1700 r.p.m. for 30 minutes in a refrigerated centrifuge at $-5°C$. The supernatant containing no haemagglutinin was discarded. The precipitate was washed with one-tenth the original volume of chilled 0.02 M phosphate buffer (pH 6.2) and again centrifuged at 1700 r.p.m. for 30 minutes at $-5°C$. The washed precipitate was then suspended in 0.3 M phosphate buffer (pH 7.0), the volume of buffer used being half the original volume of infected fluid. The suspension was kept at room temperature (19°-27°C) for one hour and then centrifuged for 15 minutes at room temperature. The supernatant was then centrifuged at 40 000 r.p.m. in a Spinco ultracentrifuge to deposit all the virus. This deposit was then suspended in 0.1 M phosphate buffer (pH 7.0) and constituted the purified virus.

Aluminium phosphate method

Highly purified virus could be obtained by the aluminium phosphate gel method described by Miller & Schlesinger (1955). However, the method was not suited for purification of the virus on a large scale for vaccine production. Therefore the technique was modified, using only the first step described by the authors for the purification of the virus. The method employed was as follows.

The aluminium phosphate gel required for 1000 ml of allantoic fluid was prepared fresh by mixing 80 ml (diluted to 1000 ml) of a 16% solution of trisodium phosphate and 80 ml (diluted to 1000 ml) of 10%

aluminium chloride solution. The quantities were proportionately reduced when smaller amounts of allantoic fluids were used. The resultant precipitate was centrifuged and washed twice with 0.125 M phosphate buffer (pH 6.0-6.2). The washed precipitate was then suspended in the allantoic fluid and mixed well. It was kept at 4°C for 90 minutes with occasional shaking, at the end of which time it was centrifuged and the supernatant containing no haemagglutinin was discarded. The aluminium-phosphate–virus complex was then suspended in 0.25 M phosphate buffer (pH 7.6-8.0), kept at room temperature for one hour with occasional shaking and then centrifuged at 3000 r.p.m. for 10 minutes. The clear supernatant represented the purified virus.

The phosphate buffer used for elution of the virus from the gel-virus complex varied with the strain of virus, e.g., for PR8, FM1 and Crawley, 0.25 M buffer of pH 8.0 was used; while for PAR and Gilbert strains buffer of pH 7.6 was used.

In the case of the B strains the method of adsorption of the virus had to be modified. Equal parts of allantoic fluid and 0.125 M buffer (pH 8.0) were mixed and the final pH adjusted to 8.0. Aluminium phosphate gel washed in 0.063 M buffer (pH 8.0) was then added to the diluted infected fluids and kept at 4°C for 90 minutes. The gel-virus complex was then centrifuged down and the deposit washed in 0.063 M buffer (pH 8.0) before elution of the virus.

The total nitrogen content of the final product, using a single cycle of adsorption with aluminium phosphate and elution, was found to be rather high compared with that obtained by the ultracentrifugation and methanol precipitation methods. Therefore, a second cycle of adsorption with aluminium phosphate and elution was also tried. For this purpose the material obtained after first treatment was diluted with an equal volume of distilled water and the pH brought to 6.0-6.2. It was then adsorbed with aluminium phosphate gel previously washed with 0.125 M phosphate buffer (pH 6.2). Then the virus was eluted into 0.25 M phosphate buffer.

Zinc hydroxide method

The method described by Newton & Bevis (1959) was modified and used. Zinc hydroxide (1%) was freshly prepared by adding 7 M ammonium hydroxide to 0.1 M zinc acetate solution until the pH was 8.5. The precipitate of zinc hydroxide was washed twice and resuspended with distilled water to the original volume. The suspension was added to

allantoic fluids (0.5 ml per ml of allantoic fluid), shaken well and kept at 4°C for 60-90 minutes, when all the virus was adsorbed by zinc hydroxide. The precipitate was then centrifuged at 3000 r.p.m. for 5 minutes and the supernatant containing no haemagglutinin was discarded. The precipitate was then suspended in 0.25 M phosphate buffer (pH 7.6) and kept in a water-bath at 37°C for 2 hours, by which time the virus eluted into the buffer. The supernatant obtained by centrifuging the suspension represented the purified virus.

Haemagglutinin titration

The method used was the same as that described by Veeraraghavan.[1]

Virus titration

Virus titrations were done in screw-capped tubes using minced chorio-allantoic membrane according to the method described by Veeraraghavan et al.[2] The culture medium employed was a modification of that reported by Eaton et al. (1960). This method was found to be comparable to egg titration in sensitivity. All the purified materials were made up to the volume of the original allantoic fluid and tenfold dilutions in Hanks' basal salt solution inoculated in 0.1-ml amounts into 0.9 ml of the medium. The cultures were rotated in a roller drum at 37°C for 96 hours and then tested for presence of virus by the haemagglutination test. Four or five tubes were used for each dilution and the virus titres—i.e., tissue culture infective doses ($TCID_{50}$) per ml—were calculated by the method of Reed & Muench (1938). These were compared with the virus content of the starting material.

Determination of purity

The total nitrogen was estimated by the micro-Kjeldahl procedure. The purity of the product obtained was assessed by calculating the haemagglutinin (HA) and $TCID_{50}$ per mg of nitrogen. The values have been expressed in logarithms.

RESULTS

Haemagglutinin recovery

The range of haemagglutinin yields obtained with the various methods on three different batches of material are summarized in Table 1. The results showed marked consistency.

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[1] See the article on page 687 of this issue.
[2] See the article on page 711 of this issue.

TABLE 1

YIELD OF HAEMAGGLUTININS WITH DIFFERENT METHODS

Virus	Percentage yield of haemagglutinins by:				
	Ultra-centrifugation	Red cell adsorption	Methanol precipitation with ultra-centrifugation	Aluminium phosphate	Zinc hydroxide
PR8	100	100	100	100	100
FM1	100	100	80-100	100	100
PAR	100	94-102	69-75	86-100	100
Gilbert (LEP)	70-80	40-60	75-90	75-100	75-100
Gilbert (HEP)	90-100	90-100	75-80	75-100	100
Crawley	100	66-80	75	90-95	100

It was found that ultracentrifugation and the zinc hydroxide method gave 100% yields with all the strains studied except Gilbert (LEP) virus, for which the recovery ranged from 70% to 100%. The aluminium phosphate method gave similar results except that the yields with Crawley, PAR, Gilbert (LEP) and (HEP) viruses varied from 75% to 100%. The methanol precipitation method came next with slightly lower yields. The red cell adsorption method gave good results with PR8, FM1, PAR and Gilbert (HEP) viruses. But the recovery was poor with Crawley and Gilbert (LEP) viruses.

Virus recovery

All the purified materials were made up to the original volume of the allantoic fluid. The $TCID_{50}$ per ml of the original material and the purified

fraction obtained with each of the methods employed was determined. As the pattern of haemagglutinin recovery in three sets of experiments was found to be consistent, virus titrations were carried out on only one of the batches. The results are given in Table 2.

The data indicated that the virus recovery was nearly the same with PR8, FM1, Crawley, PAR and Gilbert (HEP) strains employing the ultracentrifugation, red cell adsorption, methanol precipitation and aluminium phosphate methods. The virus titres were, however, lower with the zinc hydroxide method. With Gilbert (LEP) virus the virus titres were highest with the aluminium phosphate method, the titres obtained with the methanol precipitation, red cell adsorption, zinc hydroxide and high-speed centrifugation methods being next in the order mentioned.

TABLE 2

INFLUENZA VIRUS CONTENT WITH DIFFERENT METHODS

Virus	Log $TCID_{50}$/ml of original allantoic fluid	Log $TCID_{50}$/ml of material obtained after purification by:				
		Ultra-centrifuga-tion	Red cell adsorption	Methanol precipitation with ultra-centrifuga-tion	Aluminium phosphate	Zinc hydroxide
PR8	5.75	5.75	5.75	5.50	5.75	5.24
FM1	5.50	5.50	5.50	5.50	5.50	5.50
PAR	7.83	7.17	6.83	6.83	6.83	5.17
Gilbert (LEP)	5.50	3.50	3.75	4.75	5.24	3.75
Gilbert (HEP)	8.40	7.75	7.75	7.50	7.50	6.50
Crawley	6.50	6.24	6.24	6.24	6.00	6.24

TABLE 3

HAEMAGGLUTININ AND VIRUS CONTENT PER mg OF NITROGEN WITH DIFFERENT METHODS

Virus	Original virus		Ultra-centrifugation		Red cell adsorption		Methanol precipitation with ultra-centrifugation		Aluminium phosphate		Zinc hydroxide	
	$\log$ HA/mg N	$\log$ TCID$_{50}$/mg N	$\log$ HA/mg N	$\log$ TCID$_{50}$/mg N	$\log$ HA/mg N	$\log$ TCID$_{50}$/mg N	$\log$ HA/mg N	$\log$ TCID$_{50}$/mg N	$\log$ HA/mg N	$\log$ TCID$_{50}$/mg N	$\log$ HA/mg N	$\log$ TCID$_{50}$/mg N
PR8	2.81	5.98	4.26	7.43	3.70	7.21	5.29	7.21	3.52	6.70	2.89	5.54
FM1	2.16	5.68	3.66	6.18	3.19	6.69	3.84	7.35	2.89	6.41	2.24	5.76
PAR	3.15	8.57	4.63	9.39	4.27	8.69	4.66	9.08	3.77	8.22	3.24	6.00
Gilbert (LEP)	2.42	5.51	3.79	5.00	3.27	4.91	3.85	6.28	3.12	5.95	2.51	3.86
Gilbert (HEP)	3.66	8.58	5.01	9.40	4.77	9.02	4.96	9.10	4.70	8.33	3.76	6.77
Crawley	3.26	6.88	4.81	8.17	4.30	7.75	4.74	8.11	3.82	7.06	3.36	6.72

Nitrogen content

The haemagglutinin and TCID$_{50}$ per mg of nitrogen with the different methods are given in Table 3.

The results with ultracentrifugation alone and with methanol precipitation were similar. Next in order were the methods using red cell adsorption and aluminium phosphate precipitation. The zinc hydroxide method gave the poorest results.

Purification by methanol precipitation alone

In view of the relatively high purity of the material obtained after methanol precipitation and ultra-centrifugation, experiments were undertaken to determine the effect of methanol precipitation alone. The results are given in Table 4.

It was found that with methanol precipitation alone the yields of haemagglutinin and virus were

TABLE 4

RESULTS WITH METHANOL PRECIPITATION ALONE AND WITH ULTRACENTRIFUGATION

Virus	Original virus				Methanol alone				Methanol with ultracentrifugation			
	HA/ml	$\log$ HA/mg N	$\log$ TCID$_{50}$	$\log$ TCID$_{50}$/mg N	HA/ml	$\log$ HA/mg N	$\log$ TCID$_{50}$	$\log$ TCID$_{50}$/mg N	HA/ml	$\log$ HA/mg N	$\log$ TCID$_{50}$	$\log$ TCID$_{50}$/mg N
PR8	256	2.13	5.63	5.36	256	3.41	5.61	6.62	256	3.66	5.11	6.36
FM1	2 048	3.65	4.83	5.18	2 048	4.69	4.80	6.18	2 048	4.99	4.20	5.77
PAR	256	2.81	6.38	6.79	256	3.78	6.17	7.55	256	3.96	5.89	7.44
Gilbert (LEP)	256	2.49	6.83	6.91	256	3.78	6.50	7.88	192	4.07	5.50	7.29
Gilbert (HEP)	4 096	3.85	6.50	6.74	4 096	4.99	6.38	7.76	3 072	5.17	6.17	7.85
Crawley	2 048	3.69	4.63	5.01	1 536	4.56	4.50	5.75	1 536	4.70	4.42	5.93

TABLE 5

RESULTS WITH TWO CYCLES OF ALUMINIUM PHOSPHATE TREATMENT

Virus	Original virus				Aluminium phosphate, 1st cycle				Aluminium phosphate, 2nd cycle			
	HA/ml	$\log$ HA/mg N	$\log$ $TCID_{50}$	$\log$ $TCID_{50}$/mg N	HA/ml	$\log$ HA/mg N	$\log$ $TCID_{50}$	$\log$ $TCID_{50}$/mg N	HA/ml	$\log$ HA/mg N	$\log$ $TCID_{50}$	$\log$ $TCID_{50}$/mg N
PR8	256	2.13	5.63	5.36	256	2.71	5.63	5.93	256	3.64	5.60	6.83
FM1	2 048	3.65	4.83	5.18	2 048	4.28	4.81	5.77	2 048	5.17	4.63	6.48
PAR	256	2.81	6.38	6.79	256	3.48	6.08	7.16	256	4.09	6.00	7.68
Gilbert (LEP)	256	2.49	6.83	6.91	256	3.18	6.80	7.57	192	3.92	6.80	8.44
Gilbert (HEP)	4 096	3.85	6.50	6.74	4 096	5.62	6.50	7.45	4 096	5.22	6.50	8.05
Crawley	2 048	3.69	4.63	5.01	2 048	4.32	4.63	5.64	1 536	5.14	4.50	6.45

nearly 100% with all the strains tested except Crawley. On ultracentrifugation there appeared to be a fall in the $TCID_{50}$. But a fall in the haemagglutinin recovery was noticed only in the case of Gilbert (LEP) and (HEP) viruses. The slight increase found in the haemagglutinin per mg of nitrogen was not accompanied by a corresponding rise in the $TCID_{50}$ per mg of nitrogen.

Purification by two cycles of adsorption with aluminium phosphate and elution

Experiments were undertaken to determine whether the yield of haemagglutinin and $TCID_{50}$ per mg of nitrogen with the aluminium phosphate method could be improved by employing a second cycle of adsorption and elution. The results are given in Table 5.

It was found that with two cycles of aluminium phosphate adsorption and elution the material obtained was much purer. The haemagglutinin and $TCID_{50}$ contents per mg of nitrogen were generally higher than those obtained by methanol precipitation followed by ultracentrifugation.

DISCUSSION

It was found that ultracentrifugation at 40 000 r.p.m. for 30-60 minutes deposited all the hae-magglutinins present in PR8, FM1 and Crawley

strains, but this was not the case with the Asian strains. Centrifugation at 40 000 r.p.m. for 90 minutes sedimented practically all haemagglutinins from PAR and Gilbert (HEP) strains. But the recovery was only 70%-80% in the low egg passage material of the Gilbert strain. Employing centrifugation at 40 000 r.p.m. for 90 minutes it was found that the recovery of virus with all the strains tested except Gilbert (LEP) strain was very good. The haemagglutinin and $TCID_{50}$ per mg of nitrogen with this method were as good as with either methanol precipitation or ultracentrifugation.

The haemagglutinin recovery with the red cell adsorption method was nearly 100% with the PR8, FM1, PAR and Gilbert (HEP) strains. The result was not so good with the Crawley strain and was poor with Gilbert (LEP) virus. The virus titres of the purified materials were as good as those obtained by ultracentrifugation, the results with Gilbert (LEP) virus being slightly better. The titres were also as good as those obtained by methanol precipitation followed by ultracentrifugation. The haemagglutinin and $TCID_{50}$ per mg of nitrogen were, however, poorer than those obtained by the above two methods but were greater than those with single-cycle aluminium phosphate or the zinc hydroxide method.

It was found that with methanol precipitation alone and elution into 0.3 M phosphate buffer, the yields of haemagglutinin and virus were 100% with

the strains tested except in the case of Crawley. Ultracentrifugation led to a fall in the virus titre. The haemagglutinin and $TCID_{50}$ per mg of nitrogen were greater than that obtained by single-cycle aluminium phosphate method.

With the aluminium phosphate method recoveries of haemagglutinins were as good as those obtained by ultracentrifugation. This method was better than the red cell method in that the yield with Gilbert (LEP) virus was much greater. The virus recoveries compared favourably with those obtained by the ultracentrifugation, methanol precipitation with high-speed centrifugation and red cell adsorption methods in that the titre with Gilbert (LEP) virus was better. But the haemagglutinin and $TCID_{50}$ per mg of nitrogen obtained after one cycle of adsorption were poorer than those using the other three methods. However, when the material was subjected to a second cycle of purification, the haemagglutinin and $TCID_{50}$ content per mg of nitrogen could be considerably improved. Actually they were better than the results obtained with methanol precipitation with ultracentrifugation.

The zinc hydroxide method as employed gave very good haemagglutinin recoveries with all the strains tested except Gilbert (LEP) virus, for which the yield ranged from 75% to 100%. The virus titres were, however, lower than those obtained by the other methods. The haemagglutinin and $TCID_{50}$ per mg of nitrogen were the poorest, indicating that the degree of purification obtained was of a low order.

CONCLUSIONS

As mentioned earlier the methods chosen were those which could be used in the large-scale production of influenza vaccine. In certain cases they have been modified to suit this purpose. The aim was to recover by simple methods a relatively pure antigen without much loss in the haemagglutinin and virus contents during processing. Considered from this angle the following conclusions seem warranted.

The zinc hydroxide method as employed did not give the degree of purification required and was, therefore, considered to be of not much value. The red cell adsorption method gave very good results with standard as well as the Asian strains which had undergone several egg passages. But, with low egg passage Asian strains the haemagglutinin and virus yields were poor. Apart from this, our experience during the 1957 pandemic was that in spite of all care there was a certain degree of lysis of red cells during processing, resulting in a coloured product. This fact, together with the large quantities of red cells required, rendered this method not quite suitable for large-scale production. Two cycles of aluminium phosphate treatment gave the purest product. Ultracentrifugation with or without methanol treatment was only slightly inferior. When methanol precipitation was employed, strict adherence to conditions regarding temperature, etc. was essential. The aluminium phosphate method had the advantage that it was relatively simple and could be carried out with the minimum facilities.

RÉSUMÉ

Au cours d'essais relatifs à la fabrication du vaccin antigrippal, les auteurs ont comparé diverses méthodes, le but étant d'obtenir un antigène pur, ayant perdu le moins possible de ses hémagglutinines et conservé le plus possible du virus initial. Ils ont eu recours aux techniques suivantes: ultracentrifugation; adsorption érythrocytaire et élution; précipitation par le méthanol, seule ou suivie de l'ultracentrifugation; adsorption par le phosphate d'aluminium et élution (traitement en un ou deux cycles); adsorption sur hydroxyde de zinc. C'est la méthode au phosphate d'aluminium en deux cycles qui a donné le produit le plus pur. L'ultracentrifugation avec ou sans précipitation par le méthanol permet d'obtenir des résultats presque aussi bons. Vient ensuite la technique d'adsorption par les érythrocytes.

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