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Concentration and partial purification of poliomyelitis viruses

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INTRODUCTION

During the last thirty years several publications have described methods for purifying or concentrating poliomyelitis viruses. In earlier work (e.g. Clark, Schindler & Roberts, 1930; Howitt, 1930; Rhoads, 1931; Sabin, 1932; Schaeffer & Brebner, 1933) precipitation with ammonium sulphate, heavy metals or acetone, or adsorption on alumina gel was used; however, in the absence of reliable assay methods at that time, the results obtained were of necessity no more than qualitative in nature. In more recent years Schwerdt & Schaffer (1956) have precipitated the three live virus types with methanol at pH 4–4·5 as a first step in their purification-concentration process; Taverne, Marshall & Fulton (1957) adsorbed live Type 3 virus on calcium phosphate with subsequent elution; Parke-Davis & Co. (1957) adsorbed the three types of formalin-inactivated virus on aluminium phosphate; Polson & Hampton (1957) concentrated the live viruses by pervaporation; Taylor & Graham (1958) used Dowex-1 for the purification of a live Type 2 virus; Kritchevsky & McCandless (1959) adsorbed a Type 2 strain on cholesterol; Grossowicz, Mercado & Goldblum (1960) precipitated all three live or inactivated types with divalent cobalt or other divalent metals; Levintow & Darnell (1960) used vacuum distillation as a first concentration step for live Type 1 virus; Charney (1960) precipitated all three live virus types with yeast nucleic acid at pH 3·5 at the beginning of his process; Norrby & Albertsson (1960) concentrated and purified a live Type 1 virus by partitioning between two aqueous polymer phases. Further purification or concentration of the relatively crude products obtained by the steps mentioned above was usually achieved by ultracentrifugation (Charney, 1960) or the use of cellulose ion exchangers (Hoyer, Bolton, Ormsbee, Le Bouvier, Ritter & Larson, 1958), density gradient (Roizman, Mayer & Rapp. 1958; Levintow & Darnell, 1960) centrifugations and electrophoresis (Polson, Hampton & Deeks, 1960).

The object of the work recorded here was to find a method by which large volumes of the live or formalin-inactivated virus of the three types could be concentrated 10- to 100-fold without appreciably increasing the amount of the monkey-kidney protein in the preparations. Since the method was to be used in the manufacture of a vaccine, it was desirable that the operations involved should be simple and such that sterile conditions could be maintained. The methods previously used, except precipitation with nucleic acid, did not seem to be suitable: some were technically rather difficult for large-scale operation, others required

subsequent removal of undesirable additives or achieved only concentration without purification.

Parke-Davis & Co. (1957) showed that the three formalin-inactivated virus types could be adsorbed by aluminium phosphate and that such adsorbates were antigenically highly potent. No attempts seem, however, to have been made to elute the activity from the adsorbates to prepare a concentrated, aluminium-free vaccine. The procedure below involves a modification of the adsorption conditions and provides a method for eluting the live and inactivated viruses in a concentrated and purified form.

MATERIALS

Viruses. Brunenders (Type 1), M.E.F. (Type 2) and Saukett (Type 3) strains were used.

Starting materials for concentration and purification

For work with live viruses, harvest fluids (Parker's '199' medium) from infected monkey-kidney cells grown either in suspension or in monolayers were used. These were preferably clarified by filtration through pretreated asbestos pads. Formalin-inactivated viruses were also used. It did not seem to matter whether excess formalin was neutralized with sodium metabisulphite or not. The protein content of the initial fluids was usually 5–60 μg./ml.

Aluminium phosphate

This was prepared essentially as described by Miller & Schlesinger (1955), but with 100–200 times the quantities. The precipitated aluminium phosphate was allowed to settle, and the supernatant was siphoned off. The precipitate was washed three times by resuspension in distilled water (approximately one sixth the volume of the original suspension). The final precipitate was suspended in distilled water to give an $AlPO_4$ concentration of 10–15 mg./ml. During washing of the precipitate, settling was sometimes slow. This was accelerated by acidifying the suspension to about pH 5·9 with dilute HCl. The pH of the settled suspension was then adjusted to 6·5 with NaOH, and the suspension was sterilized by autoclaving at 20 lb. pressure for 1 hr. Such suspensions have been kept for several months at 4° C. or room temperature without any noticeable changes.

METHODS

Adsorption of the antigens on to aluminium phosphate

Aluminium phosphate suspension was added to the fluid containing live or formalin-inactivated virus so that a final concentration of 0·1–0·2 mg./ml. $AlPO_4$ was obtained. The pH of the mixture was then adjusted to 5·0 with N-HCl or 10 % acetic acid.

In small-scale experiments the mixtures were agitated on a shaker for 2 hr. (for convenience in centrifuge cups) and then spun for 10 min. at 1000 to 1500 *g*. The supernatant was readily decanted and could be discarded.

In large-scale work (e.g. 20 l.) the virus-$AlPO_4$ suspensions were contained in bottles fitted with two glass tubes arranged in the same way as in wash bottles. The lower end of the longer tube touched the bottom of the bottle at its periphery, the tube being pushed in sufficiently to prevent any movement. The mixtures were stirred magnetically for 2 hr. at room temperature (only magnets covered with, e.g., silicon rubber or Teflon could be used; uncovered magnets invariably produced black sludges in the presence of aluminium or calcium phosphate). The bottles were then stored overnight at 4° C. on special stands in such a way that they were inclined at about 45°, the longer tube being directed to a point some 30° from the nadir of the bottom circle. The aluminium phosphate settled out into a small volume. The supernatant liquid was siphoned through the long tube by initially applying a little air pressure from a blowball through an air-filter connected to the short tube. By rotating the bottle slightly, the bottom end of the long tube could be placed just above the adsorbate so that only the minimum quantity of the supernatant was left in the bottle at the end of the siphoning process.

Elution of the antigens from $AlPO_4$-adsorbates

For efficient elutions it was important for the final pH of the $AlPO_4$-eluting mixture suspension to be at least 7, preferably 8. In early experiments aqueous NaOH, $NaHCO_3$, Na_2CO_3 or Na_2HPO_4 were used to secure this. Provided the final pH was sufficiently high, these agents were effective but such eluates, even after prolonged spinning at 1000 to 2000 *g*, had a slightly opalescent appearance, perhaps due to colloidal $Al(OH)_3$ or $AlPO_4$. This was avoided by using the alkalis dissolved in physiological saline instead of in water. In most of the earlier experiments Na_2HPO_4 was used, but was discarded later because eluates containing PO_4 formed precipitates when mixed with '199' medium. Since the $AlPO_4$ adsorbates always retained a certain amount of the pH 5 supernatant, which lowered the pH of the added eluting fluid, buffered solutions ($NaHCO_3$) were preferred to those of sodium hydroxide. Finally, it was known that the poliomyelitis antigens were stable for long periods in '199' medium, and this medium was therefore given preference over saline as the main constituent of the eluting fluids. The fluid finally adopted consisted of 9 parts of '199' medium and 1 part of 10 % aqueous $NaHCO_3$ (or 8 and 2 parts, respectively).

In the small-scale experiments small volumes of eluting fluid (1/5 to 1/20 of the original virus fluids) were added to the adsorbates; these mixtures were then mechanically shaken for half an hour and spun as before.

In the large-scale experiments the adsorbate with the small volume of residual supernatant was transferred from the bottle through the long tube into at least one centrifuge cup by means of air pressure (see adsorption section), after rotating the bottle until the dip tube was at the lowest point of the container. The residual supernatant was removed by spinning, elution was then carried out as described above; volumes of eluting fluid as small as 1/100 of the original virus fluid could be used.

Alternatively, the residual adsorbate suspension in the bottle was neutralized with NaOH, eluting mixture was then added and elution carried out by stirring.

Protein assays

The method of Lowry, Rosebrough, Lewis Farr & Randall (1951) was used: its sensitivity was increased when necessary by using longer absorptiometer cells.

Complement fixation assays for poliomyelitis and monkey-kidney antigens

These were performed by using drops of reagents on Perspex tiles, as described by Fulton & Dumbell (1949). Poliovirus or monkey-kidney antigens were titrated in serial twofold dilutions with the constant concentration of anti-serum found to give maximal antigen titres. The titres of antigen were the dilutions giving 50% fixation of complement.

Infectivity assays

These were performed in monkey-kidney cells by serial dilutions of virus. The end-points were read either by observing cytopathic changes in roller tubes or by observing colour changes due to the metabolism of cell suspensions, as described by Salk, Younger & Ward (1954). The titres by the cytopathic tests (C.P.E.) were on average 1·1 $\log_{10}$ units higher than those by the metabolic tests (colour). The 50% end-points were calculated by Kärber's method (1958).

D-antigen assays

These were performed as described by Beale & Mason (1962).

Concentration of antigen by dialysis against Carbowax and ultracentrifugation

These are also described in the paper by Beale & Mason (1962).

RESULTS

The concentration and purification of the three poliomyelitis virus types as judged by complement-fixation assays

In preliminary experiments, mainly with Type 1 virus, it was found that even relatively large amounts of $AlPO_4$ (10 mg./ml.) did not adsorb the virus quantitatively. Lowering the pH of the virus-$AlPO_4$ suspensions, however, caused a marked increase in virus adsorption, so that eventually much smaller concentrations of $AlPO_4$ could be used for the removal of most of the viral antigen from the suspending medium.

The results of such orientating experiments are summarized in Tables 1, 3 and 4. Adsorptions and elutions were carried out as described in the 'Methods', but in early experiments different eluting fluids were used. Table 2 shows the monkey-kidney antigen content of the various fractions of Table 1.

In these experiments 100 ml. portions of the formalin-inactivated virus suspensions were treated with graded amounts of aluminium phosphate at various pH values. The adsorbed antigens were eluted with 5 ml. of 0·2 M-Na_2HPO_4, thus effecting a 20-fold concentration by volume. Complement-fixation (CF) titrations were performed on the eluates and on the 'spent' supernatants. The latter and also the

initial fluid, had to be concentrated by ultracentrifugation in order to get sufficient antigen to fix complement.

These results show clearly the need to adsorb the three virus types at a low pH. Table 2 shows that monkey-kidney protein also is better adsorbed at a lower pH, with a detrimental effect on the purity of the eluates. This disadvantage can be largely avoided by using the smallest amount of $AlPO_4$ sufficient to adsorb all the poliomyelitis antigen.

Table 1. *Effect of pH and $AlPO_4$ concentration on the adsorption and elution of formalin-inactivated Type* 1 *poliomyelitis virus*

		Supernatants (100 ml.)			20-fold concentrates (5 ml. 0·2 M-Na_2HPO_4)	
Adsorption pH	$AlPO_4$ (mg./ml.)	Concentrated on ultra-centrifuge*	C.F.†	Approx. % C.F.	CF†	Approx. % CF
4·5	1·0	8×	< 1/1	< 40	1/4	60
	0·4	30×	< 1/1	< 10	1/5	70
	0·1	16×	< 1/1	< 20	1/5	70
5·0	1·0	17×	< 1/1	< 20	1/4	60
	0·4	6×	< 1/1	< 50	1/5	70
	0·1	11×	< 1/1	< 30	1/4	60
5·5	1·0	11×	< 1/1	< 30	1/4	60
	0·4	15×	1/1	20	1/2	30
	0·1	30×	1/2	20	1/1·5	20
6·0	1·0	10×	1/2	60	< 1/1	< 15
	0·4	15×	1/3	60	< 1/1	< 15
	0·1	17×	1/3	50	< 1/1	< 15
6·5	10	5×	1/3	170	< 1/2	< 30
	1·0	17×	1/4	70	< 1/1	< 15
	0·4	25×	1/6	70	< 1/1	< 15
	0·1	5×	1/1	60	< 1/1	< 15
Initial fluid		5×	1/1·7 = 100%		—	—

* The initial fluid and the acid supernatants were concentrated by ultracentrifugation before the complement fixation assay.

† CF = Complement-fixing poliomyelitis antigen titre.

The concentration of live viruses as judged by infectivity assays

Assays of the fractions obtained in the work so far reported had been carried out by the complement-fixation method.

Unless specific antisera to the D- and C-components are used (Roizman, Mayer & Roane, 1959) this method can give misleading results. It was, therefore, necessary to discover whether $AlPO_4$ eluates contained mainly D-component or whether a conversion of D to C had taken place during the process. The simplest way to get a rough check on this was to test the recovery of infectivity in the eluates. Experiments with the three live virus types were carried out. The results are shown in Table 5. Types 1 and 2 were eluted with a mixture of '199' medium

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Table 2. *Effect of pH and $AlPO_4$ concentration on the adsorption and elution of the monkey-kidney antigen (solutions as for Table 1)*

Adsorption pH	$AlPO_4$ (mg./ml.)	Supernatants (100 ml.) CF*	Supernatants (100 ml.) Approx. % CF	20-fold concentrates (5 ml. 0·2 M-Na_2HPO_4) CF*	20-fold concentrates (5 ml. 0·2 M-Na_2HPO_4) Approx. % CF
4·5	1·0	< 1/1	< 30	1/32	50
	0·4	< 1/1	< 30	1/16	25
	0·1	< 1/1	< 30	1/16	25
5·0	1·0	< 1/1	< 30	1/16	30
	0·4	1/1·5	50	1/1	2
	0·1	1/2	70	1/1	2
5·5	1·0	1/3	100	1/7	12
	0·4	1/4	130	< 1/1	< 2
	0·1	1/4	130	< 1/1	< 2
6·0	1·0	1/4	130	< 1/1	< 2
	0·4	1/4	130	< 1/1	< 2
	0·1	1/5	170	< 1/1	< 2
6·5	10·0	1/4	130	1/2	4
	1·0	1/6	200	< 1/1	< 2
	0·4	1/5	170	< 1/1	< 2
	0·1	1/5	170	< 1/1	< 2
Initial fluid		1/3 = 100 %		—	—

* CF = Complement-fixing monkey-protein antigen titre.

Table 3. *Effect of the pH and $AlPO_4$ concentration on the adsorption and elution of formalin-inactivated Type 2 poliomyelitis virus*

Adsorption pH	$AlPO_4$ (mg./ml.)	Supernatants (100 ml.) Concentrated on ultra-centrifuge*	Supernatants (100 ml.) CF†	Supernatants (100 ml.) Approx. % CF	20-fold concentrates (5 ml. 0·2 M-Na_2HPO_4) CF†	20-fold concentrates (5 ml. 0·2 M-Na_2HPO_4) Approx. % CF
4·5	1·0	26×	< 1/1	< 10	1/12	90
	0·4	12×	< 1/1	< 10	1/16	120
	0·1	19×	< 1/1	< 10	1/6	40
5·0	1·0	16×	< 1/1	< 10	1/12	90
	0·4	28×	< 1/1	< 10	1/16	120
	0·1	15×	< 1/1	< 10	1/12	90
5·5	1·0	19×	< 1/1	< 10	1/12	90
	0·4	17×	< 1/1	< 10	1/7	50
	0·1	24×	< 1/1	< 10	1/7	50
6·0	1·0	19×	< 1/1	< 10	1/6	40
	0·4	24×	1/1	6	1/10	70
	0·1	21×	1/6	40	1/3	20
Initial fluid		24×	1/16 = 100 %		—	—

* The initial fluid and the acid supernatants were concentrated by ultracentrifugation before the complement-fixation assay.

† CF = Complement-fixing poliomyelitis antigen titre.

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(9 parts) and aqueous 10 % $NaHCO_3$ (1 part): Type 3 was eluted with a mixture of 8 and 2 parts respectively.

The results of Table 4 show that the infectious D-components of the three virus types can be concentrated and recovered in good yield. The recovery of the Type 3 virus was sometimes found to be low when 100-fold concentrations were attempted, but 10- or 20-fold concentrations gave satisfactory recoveries.

Table 4. *Effect of the pH on the adsorption and elution of formalin-inactivated Type 3 poliomyelitis virus*

		Supernatants (100 ml.)			20-fold concentrates (5 ml. 0·2M-Na_2HPO_4)	
Adsorption pH	$AlPO_4$ (mg./ml.)	Concentrated on ultra-centrifuge*	CF†	Approx. % CF	Approx. CF†	Approx. % CF
5·0	0·4	20×	< 1/1	< 10	1/16	160
6·0	0·4	10×	1/1·5	30	1/2	20
Initial fluid		12×	1/6 = 100 %		—	—

* The initial fluid and the acid supernatants were concentrated by ultracentrifugation before the complement-fixation assay.

† CF = Complement-fixing poliomyelitis antigen titre.

Table 5. *The concentration of the three virus types as judged by infectivity (colour) assay*

Material	Volume (ml.)	$AlPO_4$ (mg./ml.)	pH of adsorption	Titre (colour)	% recovery
Type 1					
Initial fluid	1000	—	—	$10^{-5·05}$	'100'
Supernatant	1000	0·1	4·7	$10^{-3·65}$	4
1st eluate	7·5	—	—	$10^{-7·20}$	106
2nd eluate	7·5	—	—	$10^{-6·30}$	13
Type 2					
Initial fluid	500	—	—	$10^{-5·35}$	'100'
Supernatant	500	0·1	5·0	$10^{-3·60}$	2
1st eluate	5	—	—	$10^{-7·40}$	112
2nd eluate	5	—	—	$10^{-6·25}$	8
Type 3					
Initial fluid	750	—	—	$10^{-5·45}$	'100'
Supernatant	750	0·2	5·0	$10^{-4·45}$	10
1st eluate	7·5	—	—	$10^{-7·10}$	45
2nd eluate	7·5	—	—	$10^{-6·15}$	5
Type 3					
Initial fluid	750	—	—	$10^{-5·45}$	'100'
Supernatant	750	0·2	5·0	$10^{-4·25}$	6
1st eluate	75	—	—	$10^{-6·35}$	79
2nd eluate	75	—	—	$10^{-4·75}$	2

The concentration and purification of the three formalin-inactivated virus types as judged by recovery of the D-antigen

Several 100 l. batches of the formalin-inactivated Type 1 virus in which the excess formalin had not been neutralized, were concentrated 100-fold by the $AlPO_4$ process (method *a*). Samples of 100 ml. initial material were partly concentrated by dialysis against Carbowax with further concentration to 1 ml. in the ultracentrifuge (for details see Beale & Mason, 1962), (method *b*).

The D-antigen contents of the Type 1 concentrates prepared by the two methods were compared (Beale & Mason, 1962); results are shown in Table 6.

Table 6. *Comparison of D-antigen content of Type* 1 *concentrates prepared via $AlPO_4$ or by Carbowax and ultracentrifugation*

		100-fold concentrates			
Starting material		Distance of 'D'-line from antiserum cup (mm.)		Protein (method *a*)	
Batch no.	Protein (μg./ml.)	Method *a*	Method *b*	μg./ml.	% recovery
1	21	25·8	25·5	214	10
2	—	26·3	25·1	—	—
3	21	25·8	25·0	151	7
4	14	24·0	25·1	192	14
5	12	25·2	25·7	164	14
6	10	24·2	24·7	144	14
7	3	23·7	23·0	20	7

Table 7. *Comparison of D-antigen content of Type* 2 *and Type* 3 *concentrates prepared by partial concentration with $AlPO_4$ and Carbowax and ultracentrifugation, or by Carbowax and ultracentrifugation only*

	Concentrates			
	Concentration factor			Distance of 'D-line' from antiserum cup (mm.)
Starting material	Method *a*	Method *b*	Total	
Type 2	25×	4×	100×	25·0
	—	100×	100×	25·7
Type 3	4·5×	22·5×	100×	26·6
	—	100×	100×	25·9

The figures of Table 6 show that the recovery of the Type 1 D-antigen in $AlPO_4$ eluates was similar to that obtained by concentration with Carbowax and ultracentrifugation. Concentration with aluminium phosphate simultaneously eliminated some 90% of the extraneous protein. Similar concentrations were also carried out with inactivated Type 2 and Type 3 fluids. In the instances shown, most of the material was concentrated with $AlPO_4$ 25× and 4·5× respectively; samples of these concentrates were further concentrated by method *b* for the D-line assay. The results are given in Table 7.

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Finally, it was shown that vaccine containing a mixture of all three virus antigens could be successfully concentrated.

Samples of such a vaccine (in which excess formalin had been neutralized with sodium metabisulphite) were concentrated by means of $AlPO_4$ (*a*) 10×, (*b*) 50×, (*c*) 10×, the eluate being concentrated by another factor of 10 to produce a 100-fold concentrate. The 10-, 50- and 100-fold concentrates were further concentrated by ultracentrifugation 30×, 6×, and 3×, respectively, to give a final 300-fold concentrate from each. A control sample (*d*) was concentrated 300× by means of Carbowax and ultracentrifugation. The 'D-lines' of the 300-fold concentrates were then determined for all three virus types. The protein contents of the materials before further concentration in the ultracentrifuge were also ascertained. The results of these experiments are summarized in Table 8. The recoveries of the three antigens were good, and again substantial purification from extraneous protein was achieved.

Table 8. *The concentration of trivalent vaccine*

	1st concentration		2nd concentration				
					Factor 1 ×	Protein	
Sample	Method	Factor 1	Method	Factor 2	Factor 2	μg./ml.	%
a	$AlPO_4$	10	—	1	10	10·0	15
b	$AlPO_4$	50	—	1	50	40·5	13
c	$AlPO_4$	10	$AlPO_4$	10	100	35·5	5·5
d	—	1	—	1	1	6·5	'100'

	3rd concentration					
			Factor 1 × Factor 2 ×	Distance of 'D'-line from antiserum cup (mm.)		
Sample	Method	Factor 3	Factor 3	Type 1	Type 2	Type 3
a	U.C.	30	300	25·9	27·5	28·4
b	U.C.	6	300	26·1	28·8	28·1
c	U.C.	3	300	26·3	28·7	28·3
d	C.W. + U.C.	300	300	26·1	27·9	28·9

C.W. = Carbowax. U.C. = Ultracentrifugation.

DISCUSSION

Attempts to adsorb any of the three viral antigens to any great extent on aluminium phosphate at neutrality—a system implied in the Parke-Davis patent, since no mention of pH adjustments is made—were not successful in our hands. On lowering the pH to about 5, however, adsorption became highly efficient, and even low concentrations of $AlPO_4$ (100–200 μg./ml.) removed the live or inactivated viruses almost quantitatively. Miller & Schlesinger (1955) have described a similar pH dependency for adsorption of influenza viruses on aluminium phosphate, but at a higher pH. The mechanism involved is probably one of true adsorption by the $AlPO_4$ and not of isoelectric precipitation (Steinman & Murtagh, 1959), since the replacement of 0·1 mg./ml. $AlPO_4$ by 0·5 mg./ml. Kieselguhr showed no

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appreciable removal of the Type 1 antigen from the virus fluid at pH 5. Moreover, it was shown that most of the antigen was removed by 0·1 mg./ml. $AlPO_4$ at pH 5 within 10 min. of contact, which again would suggest that isoelectric precipitation was not occurring.

The '199' medium-bicarbonate eluting mixtures are slightly hypertonic. Eluates, after neutralization of excess bicarbonate with HCl, can either be made isotonic by slight dilution with water or, if necessary, desalted by dialysis or passage through 'Sephadex'. On the other hand, an increased sodium chloride concentration has probably a stabilizing effect on the 'D-antigens' (Le Bouvier, 1959). The recovery of all three live or formalin-inactivated antigens was good. Moreover, it would seem that no, or at least no appreciable, conversion of the D- to the C-antigen occurred during the concentration process. If such a conversion had taken place to a marked extent, one would not expect to get quantitative or nearly quantitative recoveries of infectivity in the concentrates. Further, the *yield of antigen as measured by the 'D-line' technique was again substantially* quantitative. Since the antiserum used in the latter test contained, apart from the anti-D-component, also some anti-C-antibody, one would have expected the appearance of a 'C-line' if the concentrates had been at all rich in the C-component. This never occurred. That the absence of 'C-lines' was not due to insufficient anti-C in the antiserum could be shown by the fact that concentrates after heating to 50° C. for 3–10 min. gave rise to well defined 'C-lines'.

Aluminium phosphate, when used in the described manner, adsorbs the viral nucleoprotein more specifically than the monkey-kidney protein component, thus increasing the virus:protein ratio roughly 10-fold. Further purification can be achieved by washing the $AlPO_4$-antigen adsorbate with saline or '199' medium previously adjusted to pH 5. Alternatively, eluates can be readsorbed on fresh $AlPO_4$ and re-eluted, as shown in Table 8, sample *c*. Neither method leads to any noticeable loss of antigen.

The method can therefore be used either to concentrate poliovirus antigen or to purify it.

SUMMARY

1. Conditions are described under which the three types of live or formalin-inactivated poliomyelitis viruses can be concentrated 10–100 times in good yield, with elimination of approximately 90 % of the extraneous protein normally present in poliovaccines.

2. It was shown that 0·1–0·2 mg./ml. $AlPO_4$ almost quantitatively adsorbed the antigens, provided the adsorptions were carried out at a pH round about 5. Re-elution of the antigens occurred readily at pH 7–pH 8.

3. No noticeable conversion of the 'D-antigen' to the 'C-antigen' occurred during the concentration.

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