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Antigenic properties of the envelope of influenza virus rendered soluble by surfactant-solvent systems

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SUMMARY

Dissociating chemical treatments employing surfactant-solvent systems were applied to purified influenza A and B viruses to obtain viral preparations possessing a significantly higher or lower haemagglutinating activity than the intact virus. All preparations, whether with high or low haemagglutinating activity, with the exception of envelope protein solubilized by Triton X-100, were significantly lacking in the ability to excite the formation of haemagglutination-inhibiting and virus-neutralizing antibodies in inoculated ferrets. In contrast to other treatments, Triton X-100 treatment of virus significantly enhanced the antigenicity of viral protein as judged by virus neutralization and haemagglutination inhibition tests. Yet the haemagglutinating activity of the envelope protein solubilized with Triton X-100 was about 1 % that of the intact virus. Results suggest that the correlation assumed to exist between the haemagglutinating activity of influenza virus and its ability to excite the formation of humoral antibodies is coincidental. Another important point is that the specific antigenicity of viral protein may be lost or enhanced owing to effects, other than solubilization, by surface-active agents.

INTRODUCTION

In the present report evidence will be presented suggesting that the correlation assumed to exist between the haemagglutinating activity of influenza virus and its ability to excite the formation of humoral antibodies is coincidental. Evidence will also be presented to show that if viral protein is rendered soluble by surfactants this may result in loss or enhancement of specific viral antigenicity as judged by haemagglutination inhibition and virus neutralization tests. This phenomenon will be discussed with reference to the various biological actions of surfactants (Elworthy, Florence & Macfarlane, 1968). It has been reported that the specific biochemical or biological activity of certain biological membranes rendered soluble by surfactants may be retained, lost or enhanced (Swanson, Bradford & McIlwain, 1964; Bradford, Swanson & Gammack, 1964; Bonsall & Hunt, 1966). Limited investigations of a similar nature have been made with influenza viruses, though there has been continued interest in the antigenicity of the envelope components rendered soluble by surfactants. In general, the application of dissociating chemical treatments to influenza virus has yielded haemagglutinating and non-haemagglutinating antigens which tend to be variable over wide limits in structure, com-

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position and antigenicity (Cleeland & Sugg, 1964; Hobson, 1966; Webster & Laver, 1966; Rubin, Pierzchala & Neurath, 1967; Neurath, Rubin & Pierzchala, 1967; Neurath, Rubin & Hartzell, 1969).

METHODS

Virus

The A2/Singapore/1/57 and B/England/101/62 influenza virus strains stored at $-60°$ C. were used in this study. Pools of crude allantoic virus were prepared by inoculating allantoically 10- or 11-day chick embryos with virus diluted 10^{-3} in buffered saline; each embryo was inoculated with 0·2 ml. of diluted virus. After 48–72 hr. incubation at 35° C. the eggs were chilled at 4° C. and the allantoic fluids harvested, pooled and stored at 4° C. until the purification and concentration of virus.

Buffered saline (BS)

The solution designated buffered saline consisted of 0·85 % NaCl buffered with $NaH_2PO_4.2H_2O$ and $Na_2HPO_4.2H_2O$ (0·1 M) at pH 7·2.

Haemagglutination (HA) tests

These tests were done by the 'pattern' method in WHO haemagglutination trays (WHO, 1959). Serial twofold dilutions of virus were made in 0·25 ml. volumes of BS and then equal volumes of a 0·5 % suspension of fowl, human (group O) or guinea-pig erythrocytes were added. Readings were made after 1 hr. and end-points determined by the standard method of interpolation. Titres were expressed in terms of haemagglutinating units (HAU) per 1 ml. of undiluted virus suspension.

Purification and concentration

Pools of crude allantoic virus were clarified by centrifugation at 2000 rev./min. in the MSE-Magnum refrigerated centrifuge for 10 min. Virus was adsorbed from the allantoic fluid at room temperature by addition of Fe_2O_3 (Warren, Neal & Rennels, 1966; Larin & Gallimore, 1971). The suspension was shaken for 30 min. with the use of a Griffin flask shaker and the virus–iron oxide complex was sedimented by centrifugation at 2000 rev./min. for 5 min. Virus was eluted at room temperature and concentrated tenfold by suspending the virus–iron oxide complex in one-tenth the original volume of 10 % Na_2HPO_4 (pH 8·9). This suspension was shaken for 30 min. at room temperature and the iron oxide separated from the eluted virus by low-speed centrifugation. To avoid the effects of excess electrolyte on the solubilization of the viral envelope by surfactants, Na_2HPO_4 was crystallized out by chilling at 4° C. The virus was then sedimented by centrifugation at 60,000 *g* for 2 hr. in an MSE Superspeed 50 centrifuge. The virus pellet was taken up in a volume of BS or de-ionized water equivalent to the original volume of the eluent (10 % Na_2HPO_4). Since the determination of an overall purification factor can be hampered by the extreme lability of highly concentrated virus preparations (Pepper, 1967), the calculations to determine purification factors were made on HA titres immediately before and after purification.

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Assuming a particle/HAU ratio of 10^7, the virus concentrates contained $10^{10\cdot4}$ (A2) and $10^{9\cdot7}$ (B) virus particles/μg. protein. The purified viral concentrates (Table 1) designated as virus particles (VP) were used for the preparation of viral antigens within 24–48 hr.

Table 1. *Purification factors for concentrates of influenza virus used for preparation of viral antigens*

Virus strain	Viral material	HAU/μg protein (Lowry)	Purification factor
A2/Singapore/1/57	Allantoic fluid	28·2	96·8
	Virus concentrate	2730·6	
B/England/101/62	Allantoic fluid	2·9	176·6
	Virus concentrate	512·0	

Preparation of viral antigens

VP suspensions were divided into the appropriate number of aliquots for use in parallel studies, intact or treated chemically. All these materials, before being used as ferret inocula, were treated with formalin to inactivate residual virus. VP suspensions for treatment with Tween 80-ether were made in BS. For other treatments, VP suspensions were made in de-ionized water or as otherwise indicated in the appropriate sections below.

Tween 80-*ether* (*T 80*-E) *treatment*

The following time-schedules were used for the solubilization of VP envelope with Tween 80-ether at room temperature:

Tween 80	Ether
40 min.	2 hr.
2 hr.	6 hr.
6 hr.	18 hr.

VP suspension were mixed continuously in sealed Erlenmeyer flasks for the required times with Tween 80 at a final concentration of 0·1 % (v/v). Then equal volumes of di-ethyl ether were added to the suspensions and agitation was continued. The material was then centrifuged at 800 *g* in a refrigerated centrifuge. The aqueous phase was collected and cleared of ether by bubbling through nitrogen. The materials obtained by this treatment were designated VP/T 80-E.

Sodium dodecyl sulphate (*SDS*) *treatment*

SDS was added to VP suspension to a final concentration of 1 % (w/v). The suspension was shaken for 15 min. at room temperature and then centrifuged at 60,000 *g* for 2 hr. The supernatant obtained by this treatment was designated VP/SDS.

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Combined treatment with SDS and sodium deoxycholate (DOC)

SDS and DOC were added to VP suspension to final concentrations of 0·0015% (w/v) and 0·006% (w/v), respectively. The mixture was stirred with a magnetic stirrer for 30 min. at 37° C. and then centrifuged at 60,000 *g* for 2 hr. The supernatant obtained was designated VP/SDS-DOC.

Butanol treatment

VP suspension in 0·05 M tris-HCl buffer (pH 7·7) containing 0·001 M EDTA was mixed with 1-butanol in proportions of 1·5:1. The mixture was kept at 4° C. for 30 min. and stirred intermittently with a glass rod. The butanol-aqueous phases were separated by centrifugation in a refrigerated centrifuge at 8000 *g* for 5 min. The butanol treatment of the aqueous phase was repeated twice more as described and the final aqueous phase was designated VP/butanol.

Triton X-100 treatment

Triton X-100 (polyoxyethylated *tert*-octylphenol) was added to VP suspension in tris buffer (pH 7·5) to a final concentration of 5% (v/v). The mixture was shaken at room temperature for 5 min. and then placed at 4° C. for 10 days, after which it was centrifuged at 60,000 *g* for 2 hr. and the supernatant designated VP/TX-100.

Immunization of ferrets

Ferret inoculations

Young ferrets of both sexes (weight 700–800 g.) were bled before inoculation in pairs with the antigens just described. Each ferret was inoculated intraperitoneally with 1 ml. of the appropriate antigen. Three weeks later this inoculation was repeated and after a further 3 weeks the ferrets were killed. The pre- and post-inoculation sera and the peritoneal effusion, which were collected from each ferret, were stored at −20° C. until tested.

Titrations of antibody in ferret sera and peritoneal effusion

Haemagglutination-inhibition tests (HI). HI tests were carred out in WHO plates. Non-specific inhibitors were removed by trypsin-periodate treatment as described by Fiset (1964). Eight HAU of virus was used in all HI tests.

Virus neutralization (VN) tests in tissue culture. The method described by Hobson, Lane, Beare & Chivers (1964) was used for titrations of VN antibody. VN tests used secondary cultures of Vervet monkey kidney and the haemadsorption technique described by Shelokov, Vogel & Chi (1958). A standard virus dose of 100 haemadsorbing doses per ml. was incubated at 37° C. for 1 hr. with twofold dilutions of ferret serum or peritoneal effusion inactivated at 56° C. for 1 hr. The mixtures were inoculated into roller tubes of monkey kidney tissue culture and incubated at 35° C. for 72 hr. and then they were tested for haemadsorption. Tissue culture and virus challenge dose controls were set up with each experiment. Only complete inhibition of haemadsorption was regarded as evidence that the dilution of serum or peritoneal effusion contained VN antibody.

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Protein estimations

All protein estimations in the viral preparations just described were carried out by the method described by Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Effects of the duration of T 80-E treatment of influenza virus on its haemagglutinin titres and immunogenicity for ferrets

It has been reported that the non-haemagglutinating material of small particle size obtained with the use of trypsin or surfactants and organic solvents, though active serologically, was much less immunogenic in animals (Cleeland & Sugg, 1964; Hobson, 1966; Webster & Laver, 1966; Rubin *et al.* 1967). In view of these findings it was suggested that the integrity of the whole virus or the 70S HA sub-units obtained after ether treatment was required for quantitative maintenance of the immunogenic properties of the viral preparations (Neurath *et al.* 1967). But the question arises whether the ability of 'divalent haemagglutinin' (Choppin & Stoeckenius, 1964) to agglutinate erythrocytes necessarily implies ability to induce antibody production when injected into a suitable test animal, e.g. ferret. In this section we describe investigations into the ability of divalent haemagglutinin obtained from influenza virus A2/Singapore/1/57 by T80-E treatment for varying times to evoke production of HI and VN antibodies in inoculated ferrets. Based on preliminary experiments, the time-periods chosen for this treatment were those that increased HA titres of the treated virus with fowl, human (group O) and guinea-pig erythrocytes at several consecutive time-points during progressive disruption of the viral envelope. Although the magnitude of HAU increase at given time points varied insignificantly from experiment to experiment using the same type of erythrocytes, the kinetics of the HAU increases were similar to those shown in Table 2 for ferret inocula used in the present experiments.

Using the inocula shown in Table 2, two interesting observations were made. First, with the increased time of T80-E treatment, the inocula tended to evoke lower antibody titres in inoculated ferrets. This reduction in the capacity to stimulate the production of both HI and VN antibodies (shown in Tables 2 and 3) may be explained by partial inactivation of antigenic protein by the prolonged T80-E treatment, but owing to the small number of ferrets inoculated with each antigen this can only be a tentative observation rather than an emphatic statement. Secondly, as is seen in Table 3, ferrets inoculated with progressively increasing amounts of divalent haemagglutinin showed progressively decreasing amounts of antibody per 1000 HAU inoculated.

This finding that HI and VN antibody titres are not influenced by the concentration of divalent haemagglutinin in the inocula adds new information concerning the nature of the envelope components responsible for specific viral immunogenicity and haemagglutination. The fact that high levels of divalent haemagglutinin (inoculum VP/T80-E/3) were less immunogenic in ferrets than lower levels (inoculum VP/T80-E/1) may contradict the viewpoint that the direct effect of in-

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Table 2. *The effects of T80-E treatment on the capacity of haemagglutinating viral material to excite antibody production in ferrets*

Ferret inocula	Duration of chemical treatment		HAU/ml. of ferret inoculum with different types of erythrocyte			Mean reciprocal antibody titres of sera from ferrets inoculated with undiluted or diluted (1/5) inocula			
						HI antibody		VN antibody	
	Tween 80	Ether	Fowl	Human (group O)	Guinea-pig	Undiluted inocula	Diluted inocula	Undiluted inocula	Diluted inocula
Intact VP	Nil	Nil	8,192	12,288	8,192	320	90	1,408	420
VP/T80-E/1	40 min.	2 hr.	12,288	49,152	65,536	150	120	720	480
VP/T80-E/2	2 hr.	6 hr.	16,384	98,304	196,608	120	40	1,200	320
VP/T80-E/3	6 hr.	18 hr.	24,576	98,304	262,144	120	35	560	180

Table 3. *Reciprocal antibody titres per* 1000 *HAU* (*Rat*/1000) *with fowl, human and guinea-pig erythrocytes*

Ferret inocula	Fowl				Human (group O)				Guinea-pig			
	HI antibody		VN antibody		HI antibody		VN antibody		HI antibody		VN antibody	
	Rat/1000	%	Rat/1000	%	Rat/1000	%	Rat/1000	%	Rat/1000	%	Rat/1000	%
Intact VP	47·5	100	217·1	100	31·0	100	141·2	100	47·5	100	217·1	100
VP/T80-E/1	30·1	63	125·2	58	7·7	25	31·8	23	5·7	12	23·9	11
VP/T80-E/2	9·7	20	85·1	39	1·6	5	14·2	10	0·9	2	7·4	3
VP/T80-E/3	6·0	13	29·8	14	1·5	5	7·6	5	0·6	1	2·3	1

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fluenza virus on erythrocytes represents an important entity in the immune mechanism.

Effects of solubilization and lipid extraction on the immunogenicity of viral protein

Fractionation of envelope components of influenza virus to obtain the antigen responsible for specific viral immunogenicity has been attempted by many laboratories over a number of years. It seems that the viral envelope can easily be disrupted to progressively smaller fragments, haemagglutinating or non-haemagglutinating, but such dissociation, brought about by chemical treatment,

Table 4. *The effects of SDS, SDS–DOC, Triton X*-100 *or butanol on the affinity for erythrocytes and immunogenicity for ferrets of viral protein from A* 2/*Singapore*/1/57

	HAU/ml. of ferret inocula with different types of erythrocyte			Reciprocal titres of: HI antibody		VN antibody	
Test material	Fowl	Human (group O)	Guinea-pig	Serum	Peritoneal effusion	Serum	Peritoneal effusion
Intact VP	98,144	98,144	98,144	960	640	24,000	20,480
				960	480	19,200	15,360
VP/TX-100	1,024	512	768	1,920	1,280	30,720	30,720
				5,120	2,560	61,440	61,440
VP/SDS	< 480*	< 480*	< 480*	< 10	NT	38	20
				10	NT	80	30
VP/SDS-DOC	60	16	16	< 10	NT	50	15
				< 10	NT	< 20	17·5
VP/butanol	< 2	< 2	< 2	< 10	NT	40	15
				15	NT	120	25

NT = Not tested.

* Haemolysis in dilutions lower than 1/480.

has so far resulted in a less immunogenic viral material than the intact virus. Further progress seems to depend on dissociating viral lipoprotein without loss of immunogenicity. Yet with the present background there are no conclusive data on the chemical nature of the viral antigen responsible for evoking virus-neutralizing antibodies against influenza, nor universal agreement as to whether or not the envelope lipid is an integral part of the immunogenic antigen. This section describes a critical study of the effects of three different association colloids, the anionic SDS and DOC and non-ionic Triton X-100, on the immunogenicity for ferrets and haemagglutinating properties of the envelope proteins derived from purified VP suspensions of A 2/Singapore/1/57 and B/England/101/62 virus strains. As part of this study the envelope lipid of the above viruses was extracted with butanol and defatted viral material was examined in parallel with the intact virus.

Triton X-100 treatment has been reported from two independent laboratories to enhance the antigenic activity of an iso-antigenic lipoprotein isolated from Sarcoma I (Kandutsch & Stimpfling, 1962) and the enzyme activity of acetylcholine esterase from erythrocyte membranes (Bonsall & Hunt, 1966). We thought that

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the mechanisms postulated for these actions by Triton X-100 might also occur in the solubilization by this surfactant of envelope proteins of influenza virus. This prediction proved correct.

Tables 4 and 5 summarize the haemagglutinating activity and immunogenicity for ferrets of the intact VP and VP preparations treated with Triton X-100, SDS, SDS-DOC or butanol. Under treatment conditions a considerable or complete loss of haemagglutinating activity and immunogenicity occurred with the VP preparations treated with the anionic surfactants or defatted with butanol. In contrast, the treatment with Triton X-100 quantitatively removed the haemagglutinating activity without loss of the immunogenicity. Moreover, as is shown in Table 6, the

Table 5. *The effects of SDS, SDS–DOC, Triton X-100 or butanol on the affinity for erythrocytes and immunogenicity for ferrets of viral protein from B/England/100/62*

Test material	HAU/ml. of ferret inocula with different types of erythrocyte: Fowl	Human (group O)	Guinea-pig	Reciprocal titres of: HI antibody Serum	VN antibody Serum	VN antibody Peritoneal effusion
Intact VP	12,288	12,288	8,192	480	3,840	> 480
				160	960	50
VP/TX-100	128	256	128	1,280	4,000	> 480
				240	1,920	> 480
VP/SDS	< 480*	< 480*	< 480*	< 10	20	< 20
				< 10	30	< 20
VP/SDS-DOC	40	64	32	15	112	100
				10	200	160
VP/butanol	20	32	16	< 10	48	< 20
				< 10	60	20

* Haemolysis in dilutions lower than 1:480.

Table 6. *Immunogenicity in ferrets of inocula containing equal amounts of viral material; intact or treated with chemicals as indicated*

Influenza virus	Test material	Antibody response shown as average reciprocal titres of serum antibody: HI Titre	HI %	VN Titre	VN %
A2/Sing/1/57	Intact VP	960	100	21,600	100
	VP/TX-100	3,520	366	46,080	213·3
	VP/SDS	5	0·52	59	0·27
	VP/SDS-DOC	< 10	< 1	25	0·12
	VP/butanol	7·5	0·78	80	0·37
B/Eng/101/62	Intact VP	320	100	2,400	100
	VP/TX-100	760	237·5	2,960	123
	VP/SDS	< 10	< 3	25	1·04
	VP/SDS-DOC	12·5	3·91	156	6·5
	VP/butanol	< 10	< 3	54	2·25

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Table 7. *Reciprocal antibody titres per* 1000 *HAU* (*Rat*/1000) *ferret inoculum for intact and Triton X*-100 *treated virus*

			HI antibody response				VN antibody response			
			Serum		Peritoneal effusion		Serum		Peritoneal effusion	
Influenza virus	Test material	HAU/ml. in inoculum	Recip. titre	Rat/1000	Recip. titre	Rat/1000	Recip. titre	Rat/1000	Recip. titre	Rat/1000
A 2	Intact VP	98,144	960	9·8	560	5·7	21,600	220	17,920	183
	VP/TX-100	1,024	3,520	3,451	1,920	1,882	46,080	45,176	46,080	43,176
B	Intact VP	12,288	320	26	NE	NE	2,400	195	NE	NE
	VP/TX-100	128	760	5,938	NE	NE	2,960	23,195	NE	NE

NE = no end-point obtained (see Table 5).

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immunogenicity of viral protein of both virus strains was enhanced, this enhancement being of the order of 237·5–366 % for HI antibodies and 123–213·3 % for VN antibodies. Table 7 summarizes the haemagglutinating activity and immunogenicity of the viral protein before and after Triton X-100 treatment, demonstrating that immunogenicity persists in viral protein which has lost 99 % of its haemagglutinating activity.

DISCUSSION

Our experimental work on antigens of influenza virus has consisted of (1) an attempt to correlate the haemagglutinating power of these antigens with their ability to excite the formation of humoral antibodies, and (2) investigations on the solubilization and defatting of antigenic matter by surfactants and lipid solvents.

The principle underlying the correlation referred to under heading (1) is straightforward. Influenza virus has two distinguishable effects; *in vitro* it agglutinates some species of erythrocytes and *in vivo* it provokes the formation of humoral antibodies, haemagglutination-inhibiting and virus neutralizing. Both humoral antibodies and resistance to infection of the individual occur concomitantly, and the temptation is to believe therefore that immunogenesis is associated with the haemagglutinating power of the antigen, or, as Neurath *et al.* (1967) have suggested, that the integrity of the whole or the 70 S subunit is required for quantitative maintenance of the immunogenic properties of a viral preparation. If either of these interpretations is true, then the haemagglutinating powers of antigenic matter should be closely correlated with its ability to excite the formation of humoral antibodies in animals.

Our experiments described in this present paper failed to uphold this interpretation. Their failure must be attributed to the fact that the term haemagglutinin, 'divalent' or 'monovalent' (Choppin & Stoeckenius, 1964) merely indicates that a particulate complex adsorbs to and agglutinates some species of erythrocyte or adsorbs to erythrocytes without agglutination. Furthermore, the state of 'divalency' or 'monovalency' cannot be expressed in terms of particle size; it may be a reflexion of a preponderance of 'sites' in the particle which are capable of combining with 'receptors' of some species of erythrocytes (Choppin & Stoeckenius, 1964). As is seen in Table 2, none of the high titred haemagglutinins obtained by T 80-E treatment of VP were as immunogenically efficient as the intact VP. A most interesting result of our experiments seen in Table 2 was evidence that antigen VP/T 80-E/3, which contained considerably more 'divalent' haemagglutinin than the intact virus, was yet deficient in the ability to excite the formation of HI and VN antibodies. The most plausible explanation of this fact is that the combining sites necessary for the adsorption to and agglutination of erythrocytes are more resistant to the T 80-E treatment than the lipoprotein assemblage which determines the modality of the antibody response. On the other hand, it can be seen from Tables 4 to 7 that the Triton X-100 treatment has removed 99 % of the haemagglutinating activity of the lipoprotein assemblage without any loss in immunogenicity. Clearly, the immunogenicity and haemagglutinating activity of the lipoprotein assemblage are separable, i.e. immunogenicity persists in viral material

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that has lost most (99 %) of its haemagglutinating activity. These results suggest that the correlation assumed to exist between the haemagglutinating activity and immunogenicity of influenza virus is coincidental.

Another point of biological interest that has emerged from this present study is that solubilization of viral protein by surface-active agents is obviously important, not only as a prerequisite to some form of physical or chemical analysis, but also because the immunogenicity of the solubilized viral protein may be expected to vary greatly owing to action of surface-active agents other than solubilization.

In the case of solubilization by Tween 80–ether and anionic surfactants, SDS and DOC, the specific immunogenicity of viral protein was lost, partially or completely (Tables 2, 4, 5, 6). In contrast, the immunogenicity of viral protein solubilized by Triton X-100 was greater than that of the intact virus (Tables 4–7). Although much more work is required to elucidate the 'enhancing' mode of action of surface-active agents like Triton X-100, it would appear from our results that this goal of both theoretical and practical importance is feasible.

Our attempts to extract viral lipid by 1-butanol at 4° C. led to loss of immunogenicity as is shown in Tables 4–6. This finding suggests that the lipid may be an integral part of the envelope lipoprotein assemblage responsible for the formation of VN antibody in the recipient subject. If this is the case, then it may be that our Triton X-100 extracts contained an active water-soluble lipoprotein of the viral envelope.

The methods described in this present paper for the isolation of immunogenically active viral protein were not perfect, nor were the investigations extensive. However, they already demonstrate their usefulness in the following ways:

First, they demonstrated the coincidental nature of the correlation assumed to exist between the haemagglutinating activity and the immunogenicity of influenza virus. Therefore, solubilization and fractionation of viral protein for new improved vaccines should be monitored for specific immunogenicity rather than for HA activity.

Secondly, they showed the variability of the effects other than solubilization by surface-active agents, thus providing some basis for design of methods to produce immunogenic preparations of viral protein.

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