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Soluble antigens obtained from influenza virus by treatment with non-ionic detergent

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SUMMARY

Highly purified influenza virus was degraded using anionic and non-ionic detergents. Best results were obtained using the non-ionic detergent Triton N101. Tests showed that virus extracts contained neuraminidase and a substance that reacted specifically with rabbit antibody to virus haemagglutinin (specific serum blocking substance). Haemagglutination-inhibiting antibody was produced when virus extracts were inoculated into guinea-pigs. Immunodiffusion tests showed that extracts were complex. Host-specific material was regularly found. Under appropriate conditions S-antigen was detected as a single line pattern component. Two or more virus-specific materials were also present. One of these was probably neuraminidase and the other the specific serum blocking substance.

INTRODUCTION

It has been shown previously that influenza virus contains a group-specific 'S' or 'g' antigen (Lennette & Horsfall, 1941; Hoyle, 1945; Lief, Fabiyi & Henle, 1958), a strain-specific, haemagglutinating 'V' antigen (Fabiyi, Lief & Henle, 1958) and an antigenic neuraminidase. The neuraminidase also shows strain specificity although the serological cross-reactions of strains related by their neuraminidases do not exactly parallel the cross-reactions given by their haemagglutinins (Paniker, 1968).

In addition to these three antigens, which are generally regarded as being virus-specific, the virus also contains antigens derived from the host tissue in which it is grown (Knight, 1946; Hoyle, 1948, 1950, 1952; Smith, 1952; Smith, Belyavin & Sheffield, 1955; Howe, Lee, Harboe & Haukenes, 1957).

When the host tissue is that of the chick chorioallantoic membrane (CAM) then the host-derived antigens associated with the virus may include a heterophile antigen of the Forssman type (Kosyakov & Rovnova, 1965) and an acidic mucopolysaccharide possibly associated with the haemagglutinin (Harboe, Borthne & Berg, 1961; Haukenes, Harboe & Mortensson-Egnund, 1965). Finally Klamerth (1961) and Neurath & Sokol (1963) demonstrated the presence of host-derived

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adenosine diphosphatase and adenosine triphosphatase in purified preparations of influenza viruses.

Hoyle (1950) first showed that ether could be used to disrupt the virus and separate the haemagglutinating component from the complement-fixing internal antigen. Subsequently this method was modified and extended for use in the study of other myxoviruses. Norrby (1962) included Tween 80 in the virus-ether mixture and showed that this modification permitted more uniform results to be obtained in the disruption of measles virus. This procedure was adopted by Davenport *et al.* (1964) for the production of an effective influenza vaccine containing haemagglutinating sub-units of the virus.

Ether treatment, however, even in the presence of Tween 80, does not yield a uniform product (Choppin & Stoeckenius, 1964) and this has led to a search for more efficient agents. Laver (1963) adopted the anionic detergents, sodium deoxycholate (SDC) and sodium dodecyl sulphate (SDS) for this purpose and showed that complete virus disruption could be obtained although biological activity was not always retained. Schild & Pereira (1969) used immunodiffusion tests to show that SDS extracts of virus contained precipitable S-antigen and neuraminidase. They claimed similar results using the non-ionic detergent Non-idet P40.

Hobson (1966) combined the use of SDC with ether extraction and was able to obtain a product from the WS strain of influenza A0 which was non-haemagglutinating yet capable of blocking the haemagglutination-inhibiting (HI) activity of specific antiserum. The material was strain-specific and gave rise to HI antibodies in guinea-pigs. This work suggested that haemagglutinating activity (HA) was not essential for an influenza vaccine to be capable of evoking HI and possibly neutralizing antibody, a finding of considerable importance, as present methods of influenza vaccine standardization are based on haemagglutinin titration.

We found that the results obtained by Hobson (1966) could be reproduced with virus extracts made using non-ionic detergent. We studied our extracts in immunodiffusion tests and compared them with those made using SDC and SDS. The results of the investigation are given in this paper.

MATERIALS AND METHODS

The virus used

Strains used were: A Swine/31 (SW); A0 WS/33 (WS); A0 PR8/34 (PR8); A1 FM1/47 (FM1); A2 Jap/305/EFME/57 (EFME); A2 Rus/IKSHA/57 (IK); A2 Eng/1/66 (AE); A2 Malaysia/68 (MAL); B Eng/939/59 (B); B Eng/5/66 (B2).

With the exception of MAL, AE and B2, virus strains were supplied by Dr D. Hobson, Bacteriology Department, Liverpool University.

MAL was generously provided by Dr Lim Teong Wah, Institute for Medical Research, Kuala Lumpur, Malaysia. This strain was isolated from a patient infected with the A2 Hong Kong virus. The virus strains AE and B2 were derived from infected calf kidney tissue cultures and were a gift from Dr A. D. Kanarek of Burroughs Wellcome Research Laboratories.

On receipt all virus strains were grown in the chorioallantois of 10-day-old fertile

hen eggs. Infected allantoic fluids were harvested after 48 hr. incubation at 35° C. Appropriate pools were made and the fluids then stored in 0·1 ml. amounts in sealed glass ampoules at −60° C. A fresh ampoule was used as seed virus in each subsequent experiment.

Virus growth and purification

Seed virus was diluted to a mean 10^4 egg infective doses per ml. and propagated in chick-chorioallantois. Dilutions were made in 0·01 M phosphate buffer, pH 7·4 (PBS), containing 50 μg./ml. chloramphenicol (PBSC). Infected allantoic fluids were harvested and pooled. Pooled fluids were clarified by centrifugation at 1200 *g* for 30 min. and partially purified virus obtained by the barium sulphate absorption–elution method of Mizutani (1963). Eluates in neutralized 0·25 M sodium citrate were combined. Failure to neutralize the sodium citrate resulted in virus preparations of reduced infectivity. Virus was sedimented by centrifugation at 50,000 *g* for 60 min. and resuspended in PBSC using an MSE-Mullard ultrasonic disintegrator. The virus suspension was then clarified at 1500 *g* for 15 min. Where possible manipulations were done at 4° C. Virus purified in this way was used for inoculation into rabbits.

Final purification was by centrifugation in discontinuous sucrose density gradients. Gradients were prepared by the successive layering of 0·8 ml. volumes of 60, 50, 40, 30 and 20 % sucrose in PBSC into 5 ml. cellulose nitrate tubes and allowing diffusion to take place for several hours. Partially purified virus concentrate (1·0 ml.) was then layered on top of each gradient and centrifuged at 100,000 *g* for 15 hr. using a Spinco SW 39 L rotor.

Fractions were collected dropwise after piercing the bottom of each tube. Fractions containing virus (50–60 % sucrose) were pooled, diluted tenfold in PBSC and the virus recovered by centrifugation at 100,000 *g* for 30 min. Virus pellets were resuspended in PBSC as described previously.

Virus extracts

Extracts were made by disrupting virus with non-ionic detergents of the Triton series (Rohm & Haas Co., Philadelphia). Experiments with different Tritons showed that for this purpose Triton N 101 (Nonylphenoxypolyethoxyethanol) was to be preferred. Usually a 10 % aqueous solution of detergent was added to virus concentrates, containing not less than 10^5 HAU/ml., to a final concentration of 1 to 2 %.

After 12 hr. at 4° C. debris was removed by centrifugation at 1500 *g* for 30 min. and samples of the clarified extract diluted to a maximum Triton N 101 concentration of 0·1 %.

In the text the suffix 'T', as in SW/T, is used to denote extracts made in this way.

Non-haemagglutinating extracts were prepared also using SDC as described by Hobson (1966) and using SDS.

For electrophoresis and some immunodiffusion tests a virus pellet was dissolved in the minimum quantity of 10 % Triton N 101. Detergent was not removed, as it

was found that over a wide range of concentrations Triton N101 did not interfere in electrophoretic separations and unlike SDC and SDS did not give non-specific precipitation with antisera or other antigens in immunodiffusion tests.

Normal tissue extracts

Normal allantoic fluid (NAF) was collected from uninfected 12-day-old chick embryos. Material was clarified by centrifugation at 1200 ***g*** for 30 min., dialysed against distilled water at 4° C. for 48 hr. and dried from the frozen state. For use as antigen in immunization and serological tests 100 mg. of the dried material was dissolved in 1 ml. of sterile saline.

Normal CAM extracts (NCAM) were prepared from uninfected CAM harvested from 12-day-old chick embryos. Membranes were washed, minced at 4° C. and the minced tissue centrifuged at 1500 ***g*** for 30 min. The clear supernatant was dialysed overnight against distilled water at 4° C. and dried from the frozen state. For use as antigen in immunization and serological tests 100 mg. of dried NCAM was dissolved in 1 ml. of sterile saline.

Antisera used

Antisera were prepared in adult New Zealand white rabbits. Before immunization trial bleedings were taken from all animals. All pre-immunization sera failed to react with virus antigens, NAF and NCAM when tested by ring or gel precipitation. Moreover, no HI antibodies to any of the virus strains used were detectable.

Antiviral sera were prepared using the virus purified as described above. Virus concentrates were diluted to 10,000 HA units (HAU) per ml. and homogenized with an equal volume of Freund's complete adjuvant containing 2 mg./ml. heat-killed *Mycobacterium tuberculosis* var. *hominis* (FCA). Each rabbit received a primary injection of homogenate (1 ml.) divided over a number of intramuscular and subcutaneous sites. One month later two intravenous injections of purified virus (5000 HAU) were given 1 week apart. Serum was collected 1 week after the final injection.

Antisera to NAF and NCAM were prepared by giving primary intramuscular and subcutaneous injections of 1 ml. of antigen homogenized in FCA followed after one month by a series of weekly intravenous injections of 1 ml. of antigen without adjuvant.

The prefix 'a' as in aSW is used throughout to denote an antiserum raised against a particular virus or substance.

A sample of serum from a patient convalescent from infection with MAL was kindly donated by Dr Lim Teong Wah of the Medical Research Institute, Kuala Lumpur, Malaysia.

Serum inactivation

Non-specific serum inhibitors of virus haemagglutinin were inactivated by periodate as described by Davenport *et al.* (1964). All inactivated sera were tested for fowl haemagglutinins and positive sera absorbed with packed fowl erythrocytes.

Serum absorption

Antisera to several virus strains were absorbed exhaustively with NCAM to remove antibody to normal chick tissue antigens. Excess dried NCAM extract was added to antiserum and kept at 4° C. overnight. Particulate matter was removed by centrifugation at 25,000 *g* for 60 min. Lipid which collected on the antiserum surface during centrifugation was drawn off and discarded. Absorbed antisera gave no ring or gel precipitin reaction with NCAM but reacted with aNCAM, indicating the presence of excess absorbing material.

Absorbed antisera were stored at 4° C. after addition of sodium azide to a final concentration of 0·1 %.

Biological tests

Haemagglutination and haemagglutination-inhibition tests

These tests were done using the standard World Health Organization (1953) 'Perspex' plate method.

Strain-specific serum blocking tests

Tests for strain-specific serum blocking activity (SSB) were done essentially as described by Hobson (1966).

Neuraminidase activity

Enzymic activity was estimated from the ability of the material under test to destroy the HI property of Collocalia mucoid.

Collocalia mucoid was prepared by the method of Howe, Lee & Rose (1961) from crude swallows' nest cementing substance ('Chinese birds' nest') purchased from the Wing Lee Co., Liverpool. The available sialic acid content of the final material was estimated by the thiobarbituric acid method of Aminoff (1961) after hydrolysis of samples with 0·1 N-H_2SO_4 at 80° C. for 1 hr.

For test, mucoid was dissolved at 100 μg./ml. available sialic acid (usually 10 mg. mucoid/ml.), in 0·01 M phosphate buffer (pH 6·0). Mucoid solution (0·25 ml.) was incubated with an equal volume of 'enzyme' sample at 37° C. for 1 hr. Some of the mixture (0·25 ml.) was then diluted serially in doubling dilutions using saline buffered at pH 6·0. To each dilution 0·25 ml. indicator virus was added. The indicator virus used was influenza B heated at 56° C. for 30 min. and diluted to 32 HAU/ml. Fowl erythrocytes (0·25 ml. of a 0·5 % suspension) were then added and tests were read after 60 min. at 4° C. Scoring of results was as for HI tests. For each test a 'blank' titration was performed using mucoid incubated with an equal volume of buffer as 'enzyme'. Neuraminidase activities of samples were expressed as a percentage survival of the HI activity of the mucoid used. This figure is related to the amount of available sialic acid released on enzymic digestion of mucoid.

The method described was consistently more reproducible than simple estimation by the thiobarbituric acid reaction of sialic acid release by enzymic digestion of mucoid.

Antineuraminidase activity

The antineuraminidase titres of a number of antisera were determined. A virus suspension was prepared that contained sufficient neuraminidase in 0·25 ml. to destroy 50 % of the HI activity of a standard Collocalia mucoid solution after 1 hr. incubation at 37° C. and pH 6·0. To 0·25 ml. volumes of this preparation were added equal volumes of serially doubling dilutions of periodate-inactivated antisera. Serum-enzyme mixtures were incubated at room temperature for 30 min. after which 0·25 ml. volumes were removed and tested for neuraminidase activity as described above. Antineuraminidase titres were expressed as the reciprocal of that dilution of serum giving 50 % inhibition of the virus neuraminidase.

Immunodiffusion tests

The double diffusion technique of Ouchterlony (1948) was used. The diffusion medium was filtered 1 % Oxoid No. 1 agar in 0·85 % saline containing 0·1 % sodium azide as preservative. Reagent wells 5 mm. in diameter with centres 10 mm. apart were cut in isometric patterns in diffusion medium of a depth of 3 to 4 mm. contained in 90 mm. diameter polystyrene Petri dishes. Tests were read after 3 days development at room temperature in a saturated atmosphere. Results were recorded by photography under dark-ground illumination after gels had been washed in saline containing 0·1 % sodium azide.

Analytical disk electrophoresis

This was done in 7 % acrylamide gel as described by Davis (1964). Gels were stained with 1 % Amido-Black 10B in 7 % acetic acid and differentiated by prolonged washing in 7 % acetic acid or by electrophoresis.

Chemical analysis

Protein was estimated by the Folin–Biuret method of Sutherland, Cori, Haynes & Olsen (1949) using crystalline bovine serum albumin (Sigma) as standard.

A modified diphenylamine reaction (Burton, 1956) was used to determine 2-deoxyribose.

Absorption spectra

These were determined using an 'Optica' double-beam recording spectrophotometer, Model CF4NI.

RESULTS

Purity of virus preparations

The highly purified virus used for immunodiffusion and electrophoresis contained 50 to 80 HAU/μg. protein. No 2-deoxyribose was detected in this material and it was considered to be devoid of DNA.

Disk electrophoresis revealed a single component staining with Amido-Black 10B, which was near the junction of the large and small pore gels.

Some preparations were stained with phosphotungstic acid and examined in the

electron microscope. As shown by Corbel, Rondle & Bird (1970), little debris or non-virus-like material was seen.

Virus concentrates gave positive ring precipitin reactions when tested against antisera to the virus or to NCAM. They failed to react, however, when tested against these reagents in immunodiffusion experiments unless tests were incubated at room temperature for long periods.

These results suggested that the virus used was chemically and physically pure and was not associated with diffusible material.

Physical properties of extracts

Clarified virus extracts prepared with Triton N101, SDC and SDS contained approximately 5 mg./ml. material of virus origin. This material did not pass through 'Visking' dialysis tubing, indicating molecular weights in excess of 10,000 Daltons.

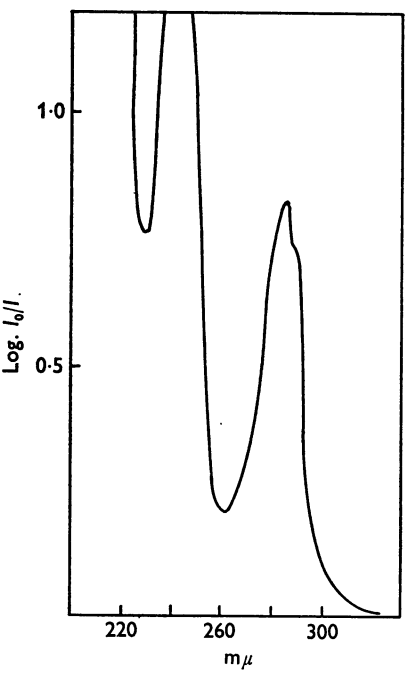

Fig. 1. Ultraviolet absorption spectrum of Triton N 101 (1/10,000 w/v).

Ultraviolet absorption spectra indicated the presence of nucleic acid and protein. Triton N101 interfered with protein detection since in addition to an absorption maximum at 240 mμ it had two absorption maxima between 280 mμ and 290 mμ (Fig. 1). The absorption maximum at 287 mμ which appears as a 'shoulder' in Fig. 1 was useful as it facilitated the detection of Triton N101 in virus extracts.

Polyacrylamide gel electrophoresis showed that virus extracts contained up to six substances staining with Amido-Black 10B. It was essential to remove SDC and SDS, although not Triton N101 before test, because extracts contaminated with anionic detergents gave a single broad fast-moving band on electrophoresis.

Extracts prepared with SDC formed thixotropic gels at 4° C. This property was not lost even after prolonged dialysis against water containing Bio-deminerolite resin (Permutit Co. Ltd.). The salicylaldehyde reaction of Szalkowski & Maeder (1952) showed that the procedure used reduced the SDC content of extracts to less than 0·01 % measured as deoxycholic acid.

Biological properties of extracts

All extracts were non-infective within the limits of the tests employed. Extracts prepared with Triton N 101 were diluted 1/100 before test because concentrations of detergent greater than 0·01 % were toxic to chick embryos.

Diluted Triton N 101 extracts were also used for HA tests. This was necessary because concentrations of detergent greater than 0·01 % lysed 0·5 % suspensions of fowl erythrocytes. The diluted extracts did not contain detectable HA.

The lytic effect on fowl cells might explain the toxicity of Triton N 101 for chick embryos.

TABLE 1. *Specific serum blocking tests with Triton N* 101 *extracts of virus*

	Reciprocal of HI titre of serum to homologous strain after treatment with:								
		A	A0		A1,	A2			B,
Serum	Saline	SW/T	WS/T	PR8/T	FM1/T	EFME/T	IK/T	MAL/T	B2/T
aSW	5,120	**240**	5,120	5,120	5,120	5,120	5,120	N/T	5,120
aWS	10,240	10,240	**240**	8,960	10,240	10,240	10,240	N/T	10,240
aPR8	10,240	10,240	10,240	**240**	10,240	10,240	10,240	N/T	10,240
aFM1	7,680	7,680	7,680	7,680	**240**	7,680	7,680	N/T	7,680
aEFME	2,560	2,560	2,560	2,560	2,560	**240**	640	1,280	2,560
aIK	5,120	5,120	5,120	5,120	5,120	1,280	**240**	2,560	5,120
aMAL	5,120	5,120	5,120	5,120	5,120	5,120	5,120	**640***	5,120
aB2	5,120	5,120	5,120	5,120	5,120	5,120	5,120	N/T	**340***

* MAL/T and B2/T were prepared from virus concentrates of approx. 20,000 HAU/ml. only.

In a few experiments Triton N 101 treated materials were centrifuged at 50,000 *g* for 1 hr. The sediment, which was small, contained some haemagglutinating material which represented, however, only a very small proportion of the original virus haemagglutinin.

Results of SSB tests with Triton N 101 extracts are shown in Table 1. They demonstrate that the serum-blocking activity of extracts was strain-specific except where two very closely related strains (EFME and IK) were studied. Similar results were obtained with virus extracts made with SDC. Other tests showed that extracts did react with heterologous antisera, but in most cases they blocked only HI antibody cross-reacting with their own strain and not HI antibody directed towards the immunizing strain. For example, aPR 8 'blocked' with WS/T retained its high titre of HI to PR 8 virus. It lost, however, its relatively lower HI titre to WS virus. The range of titres of homologous and heterologous HI antibody in the

sera used is shown in Table 2. For convenience antineuraminidase titres are included in the Table (see Discussion).

The results of SSB tests with both Triton N101 and SDC virus extracts are in agreement with those obtained by Hobson (1966) using SDC and ether.

TABLE 2. *Haemagglutination inhibition (HI) and neuraminidase inhibition (NI) titres of the antisera used*

		Reciprocal of titre with antigens:								
		A	A0		A1	A2			B	
Serum		SW	WSE	PR8	FM1	EFME	IKSHA	MAL	B	B2
aSW	HI	**5,120**	320	240	80	0	0	0	0	0
	NI	160	0	0	0	0	0	—	0	0
aWS	HI	320	**10,240**	2,560	240	0	0	0	0	0
	NI	0	80	0	0	0	0	—	0	0
aPR8	HI	240	1,920	**10,240**	160	0	0	0	0	0
	NI	0	0	160	60	0	0	—	0	0
aFM1	HI	240	320	320	**7,680**	40	0	0	0	0
	NI	0	0	80	640	0	0	—	0	0
aEFME	HI	0	0	0	0	**2,560**	1,920	80	0	0
	NI	0	0	0	0	640	320	—	0	0
aIK	HI	0	0	0	0	3,840	**5,120**	80	0	0
	NI	0	0	0	0	640	1,280	—	0	0
aMAL*	HI	40	40	40	40	160	160	**5,120**	0	0
	NI	—	—	—	—	—	—	—	—	—
aB	HI	0	0	0	0	0	0	0	**2,560**	1,920
	NI	0	0	0	0	0	0	0	480	320

0 Indicates HI or NI titres of less than 1/20.
* Human convalescent serum.
—Not tested.

TABLE 3. *Neuraminidase activity of virus preparations and Triton N101 extracts*

	A	A0		A1	A2		B	
	SW	WS	PR8	FM1	EFME	IK	B	B2
*Untreated	12·5	3·3	12·5	25	87·5	50	25	3·3
*Triton N101 extract	12·5	1·7	12·5	37·5	57·5	25	12·5	5

* Virus used contained 10^3 HAU/ml.

As with SDC extracts, Triton N101 extracts elicited HI antibodies when injected into guinea-pigs. For these tests a single intramuscular injection was given and serum samples taken 2–3 weeks later.

Neuraminidase activity was demonstrated consistently in virus extracts with the exception of those derived from WS. Extracts from this source were variable in their behaviour and on occasion no neuraminidase was found. Some results for Triton N101 extracts are shown in Table 3.

All extracts gave lines of precipitation when tested against homologous and heterologous antisera in immunodiffusion tests. Results obtained with Triton N101, SDC and SDS were similar and showed strain-specific line pattern components (lpc). However, SDS and SDC extracts frequently caused non-specific precipitation of antisera and control antigens. Moreover, patterns obtained with SDS extracts were invariably the least complex and the gelling properties of SDC extracts introduced technical difficulties. For these reasons most work was done with Triton N101 extracts which did not suffer from such disadvantages.

TABLE 4. *Number of lpc given by Triton N101 extracts of virus and antisera absorbed with NCAM*

	Antigens								
	A	A0		A1	A2			B	
Serum	SW/T	WS/T	PR8/T	FM1/T	EFME/T	IK/T	MAL/T	B1/T	B2/T
aSW	2	1	1	0	0	0	0	0	0
aWS	1	2	1	0	0	0	0	0	0
aPR8	1	1	3	1	0	0	0	0	0
aFM1	0	1	1	2	0	0	0	0	0
aEFME	0	0	0	0	2	2	2	0	0
aIK	0	0	0	0	2	2	2	0	0
aMAL*	1	1	1	1	3	3	3–4	0	0
aB	0	0	0	0	0	0	0	2	2

* Human convalescent serum.

The NAF–aNAF and CAM–aCAM control systems also gave several lpc when examined in immunodiffusion tests. It was essential, however, to use a CAM–aCAM control in tests for host materials. Some lpc present in tests on virus extracts were given by this control but were absent from NAF–aNAF or CAM–aCAM reactions. This last observation is shown in Pl. 1, fig. 6.

A typical test using virus extract and host controls is shown in Pl. 1, fig. 1. The lpc in the reaction between IK/T and aIK are labelled arbitrarily 1 to 5. Inspection shows that lpc 1 occurs in the reaction between IK/T and aNCAM and lpc 4 and 5 occur in the reaction between NCAM and aIK. This suggests that lpc 1, 4 and 5 are due to host components present in purified virus preparations. The lpc 2 and 3, however, appear only in the IK/T–aIK reaction and would seem to be virus-specific. The other reactions seen are due to host material: lpc E1 is unique to the NCAM–aNCAM reaction and lpc E2 probably represents host component in the purified virus. The lpc E2 appears to show a reaction of partial identity with lpc 3 but the significance of this is not known. The experimental design does not allow comparison of lpc E1 with lpc 1 (or E2) but was chosen to show the virus-specific nature of lpc 2 and 3.

The strain-specific nature of the virus-specific lpc is illustrated in Pl. 1, fig. 2. Here IK/T is compared with WS/T. The lpc 1, 4 and 5 of Pl. 1, fig. 1 are common to both virus extracts but lpc 2 and 3 are unique to the IK/T–aIK experiment and lpc 6 and 7 are unique to the WS/T–aWS experiment.

Further proof of the strain-specificity of IK/T components is given in Pl. 1, fig. 4 where FM 1/T is included in the experiment. The lpc 2 and 3 of the IK/T–aIK experiment are not given by WS/T or FM 1/T.

Similar results were obtained with extracts prepared from other strains. Results with PR 8/T are shown in Pl. 2, fig. 1. Here the lpc labelled 8 and 9 are probably strain-specific. Results with FM 1/T are shown in Pl. 2, fig. 2. Here lpc 10 is unique to the FM 1/T–aFM 1 system.

An attempt was made to study strain-specific antigens using antisera absorbed with NCAM to remove anti-host antibody. Results are summarized in Table 4. Absorbed antisera gave up to 3 lpc when tested against Triton N 101 extracts of homologous virus. In some cases one of the lpc was common to viruses of the same sub-group but was not found in tests involving viruses of different sub-groups, i.e. it was sub-group specific. The other lpc were strain-specific except that some cross-reactions were found between closely related viruses. For example, the strain-specific antigens in WS/T were distinct from those in FM 1/T. The virus-specific materials in PR 8/T, however, showed some cross-reaction with WS/T and FM 1/T.

Examples of tests using absorbed antisera are shown in Plate 2. Here fig. 3 shows results obtained with aPR 8 and fig. 4 results obtained with aSW. In fig. 3 the PR 8/T-absorbed aPR 8 reaction shows 3 lpc. None of these lpc are given by FM 1/T. One of the lpc (labelled 12) is given by SW/T but another (labelled 11) is unique to PR 8/T. The occurrence or absence of the other lpc in SW/T cannot be determined in this experiment. In fig. 4 the SW/T-absorbed aSW reaction shows two lpc which are not given by NCAM. These reactions are virus-specific. The SW/T–aSW reaction shows these lpc together with other lpc due to host components. Host components and other egg lpc are shown in the reaction between aSW and absorbed aSW. Clearly absorption of antisera with host material facilitated the demonstration of virus-specific materials.

Recently group- and strain-specific antigens have been demonstrated using a human convalescent serum which was free from host antibody. One result is shown in Pl. 1, fig. 3. Triton N 101 extracts of CAM-grown MAL gave at least 3 lpc when tested against the human convalescent serum VR. This serum did not react with NCAM or B/T. In other experiments one of the lpc was found to correspond to S-antigen, another was sub-group specific for influenza A 2 strains and the others were unique to MAL.

The immunological data available are in agreement with previous observations and suggest that influenza B strains possess their own group- and strain-specific antigens and are related to influenza A strains only by the presence of common host antigens. This is illustrated in Pl. 1, fig. 5, where the B/T–aB reaction gives one lpc (labelled B) not given by IK/T or NCAM.

DISCUSSION

It is thought that the virus preparations used in this work were as pure as those used by others. A possible exception was MAL. This virus had been passed only a

few times in CAM, did not grow to high titre and hence was associated with relatively more host material before purification began.

The highly purified virus preparations were agglutinated by aNCAM. This observation supports the view that host material is present in the virus surface; it differs from the results of Ananthanarayan (1954), Kroeger (1962) and Duc-Nguyen, Rose & Morgan (1966).

Purified virus did not react in immunodiffusion tests unless experiments were incubated at room temperature for prolonged periods. It is thought that in this time virus was degraded. Such an explanation would account for the results obtained by Jensen & Francis (1953), Hennisch (1960) and Nikolova & Kavaklova (1967).

Following degradation of virus a number of different host components were detected by immunodiffusion tests. For example, Pl. 2, fig. 2 shows up to five lpc common to the PR 8/T–aFM 1 and the NCAM–aFM 1 reactions. This indicates that either more than one host material is present on the virus surface or that some host material is present in the virus core or that on degradation a host component is split into a number of materials having different serologically reactive sites.

Disrupted virus extracts also contained virus-specific material. Neuraminidase and SSB activity were detected by specific tests, and immunodiffusion experiments showed several virus-specific precipitating substances. Using rabbit antisera, at least two virus-specific materials were detected in virus extracts. Detection was facilitated by the use of sera absorbed with NCAM. It was essential to use NCAM and not NAF for this purpose as the latter material lacked some of the host components found in virus extracts. It seems possible that the virus-specific precipitating substances detected by our rabbit antisera were related to SSB and neuraminidase. They were not related to S-antigen, as these sera did not contain anti-S antibody.

S-antigen was present in our virus extracts. It was detected by use of human convalescent serum. In immunodiffusion tests a single lpc appeared common to all extracts of influenza A virus tested. The finding is not illustrated in this paper but one of the lpc shown in the MAL/T–VR reaction in Pl. 1, fig. 3 is due to S-antigen.

The detection of a single S-antigen agrees with the work of Schild & Pereira (1969) but differs from that of Styk & Hána (1966), Hána & Hoyle (1966) and Styk, Hána & Sedíleková (1968), who used the Wadsworth (1957) micro-immunodiffusion technique to investigate virus disrupted with ether and SDC and tested against human convalescent serum. They described multiple S components in virus extracts together possibly with some strain-specific material.

Using virus disrupted with SDS, Schild & Pereira (1969) were unable to detect virus-specific material which did not correspond to S-antigen or neuraminidase. A substance which might have been related to HA was detected in virus disrupted by the non-ionic detergent Non-idet P 40. We suspect that, given suitable antisera, virus extracts made with non-ionic detergents give lpc in immunodiffusion tests which correspond to neuraminidase, SSB substance and S-antigen. Support for this view is given by consideration of data presented in Table 2 and Table 4.

With respect to neuraminidase, some of the virus-specific lpc correspond to the occurrence of these enzymes as determined by anti-neuraminidase tests. Strain-specific precipitating substances and strain-specific neuraminidase occur in SW and WS. Strain-specific precipitating substances and neuraminidase of restricted specificity occur in PR 8 and FM 1. In A 2 strains the neuraminidase was sub-group specific and a common precipitating substance was found.

The other virus-specific lpc corresponded to the range of HI antibodies present in the sera used. Thus in immunodiffusion tests cross-reactions were obtained with SW, WS and PR 8 and with WS, PR 8 and FM 1; there was no virus-specific lpc cross-reaction between SW and FM 1 even though there was some cross-reaction between the HI antibodies to these viruses (see Table 2). In A 2 influenza virus strains differences were found between MAL and the closely related EFME and IK. The extracts of influenza B strains examined had virus-specific materials which did not cross-react with any of the extracts of the A strains studied. The significance of other virus-specific substances detected in PR 8/T and MAL/T (see Table 4) is not known.

The work of Tyrrell & Horsfall (1954), Reginster (1965, 1966) and Hobson (1966) suggests that degradation of influenza virus HA leads to the appearance of SSB activity. It is probable therefore that the virus-specific precipitating materials apparently detected by HI antibody are those possessing SSB activity which have arisen by degradation of HA. The wider range of reactivity shown in precipitation tests as compared with the SSB tests could be explained if the materials responsible for both activities possessed strain and sub-group specific sites. The latter might well be masked in intact virus particles and might not take part in SSB tests done on virus extracts.

Ivaničova (1968) has also used immunodiffusion tests to study strain-specific materials in HA preparations of virus but it is not possible to compare our results with hers.

Most of the work reported was done with virus degraded with Triton N 101. This material did not suffer from the several disadvantages encountered with SDS and SDC. It did not give non-specific precipitates with antisera and virus extracts and apparently did not form ionic complexes or coacervations with virus antigens. Unfortunately it could not be removed from virus extracts by dialysis but on the other hand it was readily separated from virus-specific material by electrophoresis in borate buffer (unpublished observation).

A particular advantage of Triton N 101 was that it gave consistent results with all strains of influenza virus examined irrespective of strain and host tissue. Apart from the results reported for virus grown in CAM and in calf kidney tissue culture similar results have been obtained with virus grown in BHK 21 cells and in mouse lung. This suggests that Triton N 101 might be of value in the production of split virus vaccines. As shown by Corbel *et al.* (1970), this particular non-ionic detergent has a specific effect on virus degradation.

Finally these results, taken in conjunction with those of others, suggest that vaccines prepared from degraded virus do not necessarily require HA activity to be effective in the production of HI antibody. Virus-free preparations containing

SSB activity and strain-specific precipitating substances regularly induced HI antibody in guinea-pigs. Preliminary observations suggest that immunodiffusion tests might be used to assess the immunizing potency of such virus extracts.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. The IK/T–aIK reaction shows up to 5 lpc. Lpc 1 is given in the IK/T–aNCAM reaction and lpc 4 and 5 in the NCAM–aIK reaction. Lpc 2 and 3 are probably virus-specific. Other reactions, e.g. E 1, E 2, are due to CAM materials.

Fig. 2. Lpc 2 and 3 are specific for the IK/T–aIK reaction. Lpc 6 and possibly 7 occur only in the WS/T–aWS reaction. The lpc are probably strain-specific. Other lpc occur in both homologous and heterologous tests.

Fig. 3. The virus extract MAL/T reacts with the aMAL human convalescent serum VR to give at least 3 lpc. The lpc are not given by NCAM or B/T and are thus probably specific for influenza A virus.

Fig. 4. The IK/T–aIK reaction shows the lpc 2 and 3 of figs. 1 and 2. These lpc are not present in the WS/T and FM/T–aIK reactions.

Fig. 5. The B/T–aB reaction shows the lpc B, which is not given by the NCAM or IK/T–aB reactions.

Fig. 6. The NCAM–aNCAM reaction is much more complex than the NAF–aNCAM reaction. This latter test also lacks lpc present in the IK/T–aNCAM reaction.

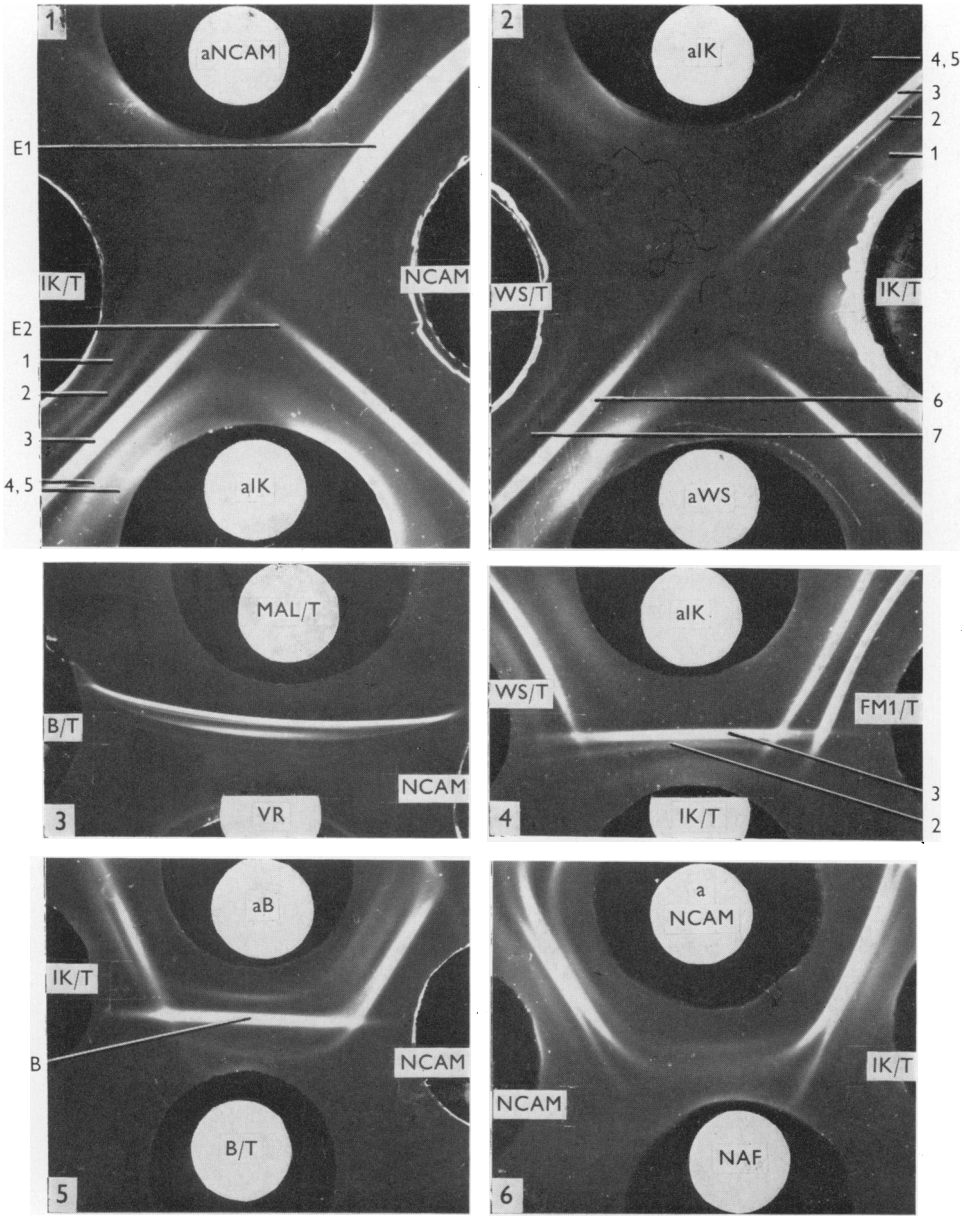
PLATE 2

Fig. 1. The PR 8/T–aPR 8 reaction shows 2 lpc (labelled 8 and 9) not present in the NCAM–aPR 8 reaction. Lpc 9 is present in the FM 1/T–aPR 8 reaction but lpc 8 is unique to the PR 8/T–aPR 8 reaction.

Fig. 2. The FM 1/T–aFM 1 reaction shows an lpc (labelled 10) which is not present in reactions involving PR 8/T or NCAM. The distribution of other lpc is marked by the complexity of the reactions.

Fig. 3. The PR 8/T–aPR 8 (absorbed with NCAM) reaction shows 3 lpc. One lpc (labelled 11) is unique to this test; another (labelled 12) is given also in the SW 8/T–aPR 8 reaction. The virus extract FM 1/T did not react with NCAM-absorbed aPR 8.

Fig. 4. The SW/T–aSW (absorbed with NCAM) reaction shows 2 lpc not given by the reaction involving NCAM. Both lpc are given by the SW/T–aSW reaction and probably represent virus-specific reactions. The aSW–aSW (absorbed with NCAM) reaction shows that absorption was complete. It also indicates the host reactions involved in the SW/T–aSW test.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E2
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4, 5
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2
1
WS/T
IK/T
6
7
aWS
MAL/T
B/T
NCAM
3
VR
aIK
WS/T
FM1/T
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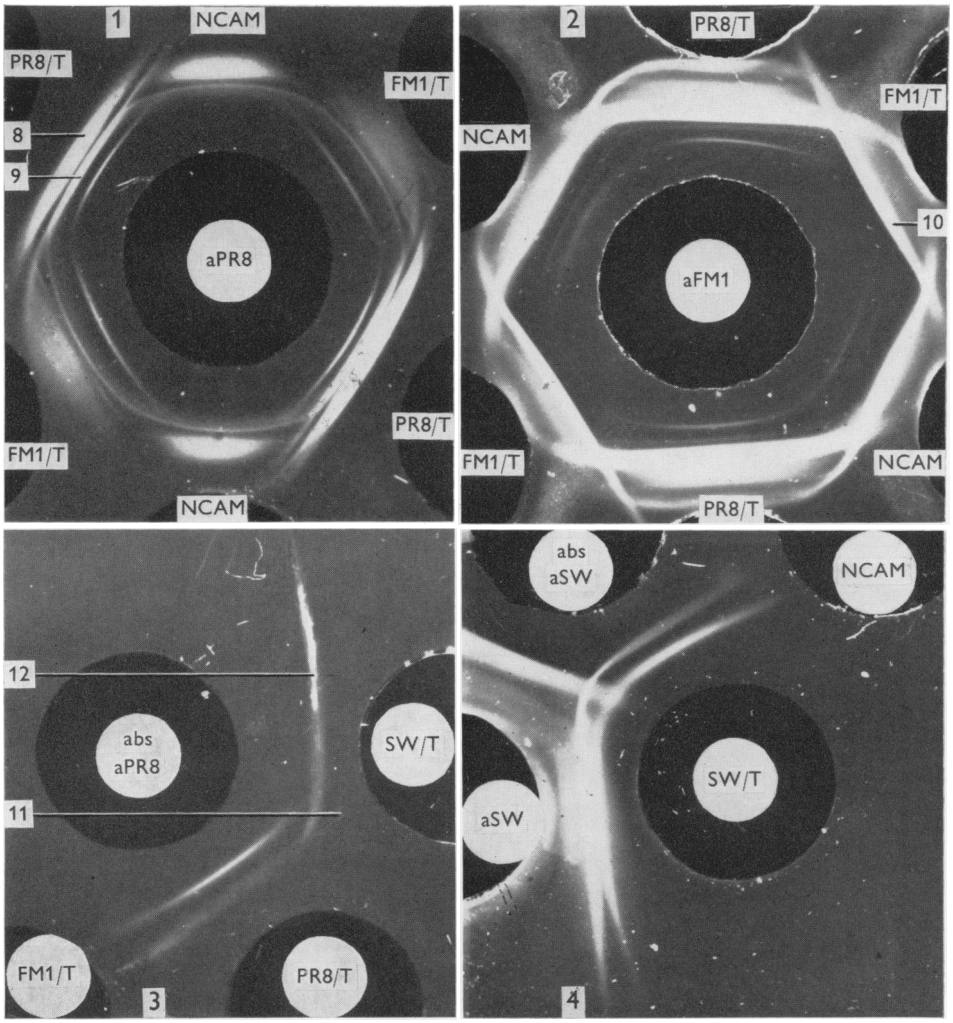
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FM1/T
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FM1/T
PR8/T
3
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SW/T
aSW
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