J. Hyg., Camb. (1971), **69**, 461
With 1 plate
Printed in Great Britain

The chemical reactions of the haemagglutinins and neuraminidases of different strains of influenza viruses

III. Effects of proteolytic enzymes

By L. HOYLE
Public Health Laboratory, General Hospital, Northampton
AND JUNE D. ALMEIDA
Virological Department, Royal Post-Graduate Medical School, Hammersmith Hospital, London

(*Received* 11 *March* 1971)

SUMMARY

The action of trypsin and pronase on the haemagglutinins and neuraminidases of eight strains of influenza virus has been examined.

The haemagglutinins of all the strains were highly susceptible to digestion by pronase but there were great variations in resistance to trypsin.

The neuraminidases of the eight strains were of three types. The neuraminidases of the A 1 strains and the DSP strain of virus A were highly susceptible to destruction by both enzymes. The neuraminidases of the PR 8 and SWINE strains showed partial resistance especially to trypsin, while the A 2 strains and the LEE strains of virus B possessed neuraminidases that were completely resistant to both trypsin and pronase.

Proteolytic enzymes released free neuraminidases from the A 2 and LEE viruses the morphology of which was different from that of neuraminidases released by detergent treatment.

INTRODUCTION

In previous studies (Hoyle 1969*a*, *b*) the resistance of the haemagglutinins and neuraminidases of eight strains of influenza viruses to treatment with chemical reagents acting on amino acids present in the active centres, and to reagents modifying the higher order structure of the virus proteins was described. The present work describes the results of treatment of the same strains with proteolytic enzymes.

MATERIALS AND METHODS

Proteolytic enzymes

Two enzymes have been used, trypsin and pronase.

Trypsin. A 10% solution of commercial trypsin (Difco) was prepared in distilled water adjusted to pH 7·5 by addition of disodium phosphate, centrifuged, and insoluble material discarded. The supernatant fluid was then desalted by

passage through a column of Sephadex G 25 equilibrated to distilled water of pH 7.5. Eluate samples were collected and those containing enzyme were pooled and 0·5 ml. volumes were frozen and stored at −20° C.

Pronase. Commercial pronase (BDH) was used and preparations made in the same way as with trypsin except that the distilled water was of pH 7·0.

Potency test of enzymes. Two ml. of a 1% solution of egg albumin in distilled water was treated for 10 min. at 37° C. with 0·05 ml of enzyme at pH 7·0 in the case of pronase and at pH 7·5 with trypsin. The mixtures were then precipitated with 5% trichloroacetic acid and the Folin colour of the supernatants measured. The trypsin preparation produced a Folin colour equivalent to 28 μg. of tyrosine, and the pronase preparation produced the equivalent of 82 μg. of tyrosine. The pronase preparation had therefore about three times the proteolytic activity of the trypsin.

Virus preparations

Virus from infected allantoic fluid was purified by two cycles of adsorption-elution from guinea-pig red cells followed by a cycle of differential centrifugation. The final deposited virus was suspended in phosphate buffered saline of pH 7·0 to a concentration of about 1% by volume. These preparations are referred to as 'virus concentrate'. Although all the preparations contained approximately the same amount of virus the haemagglutinin varied, filamentous strains giving lower titres than more highly egg-adapted spherical strains.

Electron microscopy

Virus preparations to be examined in the electron microscope were centrifuged for 1 hr. at 30,000 *g* and the deposit resuspended in a suitable volume of 1% ammonium acetate. A small amount of this was mixed with an equal volume of 3% phosphotungstic acid adjusted to pH 6·0. A drop of the mixture was then placed on a 400-mesh carbon-formvar-coated grid and excess fluid withdrawn. After drying the grid was examined immediately in a Phillips EM 300 at a plate magnification of × 60,000.

Technique of the tests

The action of the two enzyme preparations on the eight virus strains was tested under conditions of low ionic strength and for a period of 4 h. at 37 °C.

Virus concentrate (0·3 ml.) was diluted with 5·0 ml of distilled water at pH 7·0 in tests of pronase activity and at pH 7·5 with trypsin; 1·0 ml of the mixture was diluted to 3·0 ml. with phosphate buffered saline of pH 6·5 and retained as an original virus control.

To the remaining 4·3 ml. of the mixture was added 0·3 ml. of enzyme preparation and the mixture was held at 37° C. for 4 hr. It was then diluted to 12·9 ml with buffered saline pH 6·5 and centrifuged at 25,000 *g* for 1 hr. The supernatant fluid was removed and the deposited enzyme-treated virus resuspended in 12·9 ml. of buffer. The haemagglutinin titres and neuraminidase activities of the supernatant and resuspended deposit were then compared with those of the original virus.

Haemagglutination tests. These were done by the Salk method using 0·5% guinea-pig red cells.

Neuraminidase activity. An ovomucin substrate was used, prepared by diluting 250 ml. of egg white to 1 l. with distilled water, adjusting the fluid to pH 6·0 with acetic acid, centrifuging and extracting the precipitated ovomucin with 100 ml. of buffered saline of pH 6·5. After centrifugation to remove insoluble material the ovomucin solution was frozen and stored at −20° C.

In the neuraminidase tests 0·4 ml. of sample was mixed with 0·4 ml. of ovomucin solution and held at 37° C. for 30 min. Released *N*-acetyl-neuraminic acid was then determined by the Aminoff (1961) modification of the Warren technique, the coloured product being extracted with 4·0 ml. of butanol-HCl and the absorptiometer readings at 549 mμ recorded.

Table 1. *Effect of trypsin treatment on influenza viruses*

Virus	Material	Haemagglutinin titre	Neuraminidase activity	Enzyme-resistant neuraminidase
A SWINE	Original virus	8,192	0·15	
	Trypsinized virus			33%
	Supernatant	Nil	0·05	
	Deposit	Nil	Nil	
A PR 8	Original virus	4,096	0·09	
	Trypsinized virus			78%
	Supernatant	32	0·06	
	Deposit	512	0·01	
A DSP	Original virus	16,384	0·20	
	Trypsinized virus			0%
	Supernatant	512	Nil	
	Deposit	25,576	Nil	
A 1 BURCH	Original virus	8,192	0·30	
	Trypsinized virus			0%
	Supernatant	32	Nil	
	Deposit	Nil	Nil	
A 1 BRATISLAVA	Original virus	8,192	0·28	
	Trypsinized virus			0%
	Supernatant	Nil	Nil	
	Deposit	128	Nil	
A 2 TAIWAN	Original virus	8,192	0·35	
	Trypsinized virus			103%
	Supernatant	Nil	0·21	
	Deposit	256	0·15	
A 2 ENGLAND 67	Original virus	2,048	0·44	
	Trypsinized virus			141%
	Supernatant	32	0·37	
	Deposit	1,024	0·25	
B LEE	Original virus	3,072	0·29	
	Trypsinized virus			110%
	Supernatant	128	0·32	
	Deposit	8,192	Nil	

RESULTS

The results of treatment of eight strains of influenza virus with trypsin and pronase are shown in Tables 1 and 2.

Table 2. *Effect of pronase treatment on influenza viruses*

Virus	Material	Haemagglutinin titre	Neuraminidase activity	Enzyme-resistant neuraminidase
A SWINE	Original virus	4,096	0·18	0%
	Pronase virus			
	Supernatant	Nil	Nil	
	Deposit	Nil	Nil	
A PR 8	Original virus	3,072	0·08	12%
	Pronase virus			
	Supernatant	Nil	0·01	
	Deposit	32	Nil	
A DSP	Original virus	16,384	0·27	0%
	Pronase virus			
	Supernatant	Nil	Nil	
	Deposit	128	Nil	
A 1 BURCH	Original virus	4,096	0·31	0%
	Pronase virus			
	Supernatant	Nil	Nil	
	Deposit	Nil	Nil	
A 1 BRATISLAVA	Original virus	4,096	0·20	0%
	Pronase virus			
	Supernatant	Nil	Nil	
	Deposit	Nil	Nil	
A 2 TAIWAN	Original virus	2,048	0·14	107%
	Pronase virus			
	Supernatant	Nil	0·15	
	Deposit	Nil	Nil	
A 2 ENGLAND 67	Original virus	2,048	0·42	102%
	Pronase virus			
	Supernatant	Nil	0·38	
	Deposit	Nil	0·05	
B LEE	Original virus	2,048	0·32	122%
	Pronase virus			
	Supernatant	Nil	0·37	
	Deposit	Nil	0·02	

Action of proteolytic enzymes on the haemagglutinating activity

The haemagglutinating activity of all the strains was highly susceptible to destruction by pronase, but results with the less powerful trypsin preparation were variable. The haemagglutinating activity of the SWINE and A 1 BURCH strains was destroyed, with PR 8, A 1 BRATISLAVA, and the A 2 strains there was reduction of activity, while the DSP and LEE strains showed a slight increase in titre.

The significance of these results is difficult to interpret as the haemagglutination

test does not measure the amount of haemagglutinin protein present but measures the number of particles carrying more than one haemagglutinating centre. Proteolytic enzymes remove the surface projections from the virus particle but this will only result in a decrease of haemagglutinin titre if almost the whole of the projections are removed. The following experiments illustrate the difficulty.

Electron microscopy of virus treated with trypsin and pronase. Preparations of DSP virus were treated with trypsin at pH 7·5 and with pronase at pH 7·0. After 4 hr. at 37° C. the preparations were centrifuged at 30,000 *g* for 1 hr. and the deposited virus divided into two parts, one being resuspended for measurement of haemagglutinin titre and the other being suspended in 1% ammonium acetate for electron microscopy. Electron photomicrographs of the original virus and the enzyme treated preparations are shown in Pl. 1, figs. 1–3.

The original virus exhibited the 100 Å projections characteristic of influenza-virus (Pl. 1, fig. 1). Particles treated with pronase revealed a smooth membrane with no visible projections (Pl. 1, fig. 2). The overall distinctive shape of the particles was preserved. Virus treated with trypsin showed particles with an intermediate appearance, projections being present but in much smaller numbers than in intact virus (Pl. 1, fig. 3).

The original virus suspension had a haemagglutinin titre of 16,384. No reduction in titre was produced by trypsin treatment but pronase reduced the titre to 128.

Ether treatment of trypsinized virus. Preparations of DSP virus and trypsinized DSP virus were shaken with ether, centrifuged, and the aqueous phases tested for haemagglutinin content with the following result.

	Haemagglutinin titre
Original virus	16,384
Ether-treated virus	32,768
Trypsinized virus	16,384
Ether-treated trypsinized virus	128

Ether treatment of the original virus produced a slight increase in titre. Although the trypsinized virus had an unchanged haemagglutinin titre, on ether treatment the titre was reduced to less than 1%. The result suggests that the number of haemagglutinin sub-units released from the trypsinized virus was much less than from untreated virus, so that only a few multivalent aggregates could be produced.

These experiments show that changes in haemagglutinin titre of virus after trypsin treatment do not accurately reflect the action of the enzyme on the haemagglutinin protein, and cannot be used to differentiate the haemagglutinins of different strains of virus. This difficulty does not occur in studies of the action of proteolytic enzymes on the neuraminidase activity of the viruses as neuraminidase activity is shown by both monovalent and multivalent particles.

Action of proteolytic enzymes on the virus neuraminidases

The results shown in Tables 1 and 2 indicate great differences in resistance of the virus neuraminidases to proteolytic enzymes.

With three strains, the DSP strain of virus A and the two A 1 strains, the neuraminidases are highly sensitive, being totally destroyed by both trypsin and pronase.

Two strains of virus, SWINE and PR 8, show some resistance to trypsin, and the PR 8 strain also shows some resistance to pronase. With these strains a free neuraminidase is released from the virus by trypsin treatment to appear in the supernatant fluid on centrifugation, but the activity of the released enzyme is less than that of the original virus.

The two strains of A 2 virus and the LEE strain of virus B possess neuraminidases which appear to be completely resistant to proteolytic enzymes. Both trypsin and pronase split off a free neuraminidase, pronase producing a more complete release than trypsin. In all cases the total neuraminidase activity after proteolytic enzyme treatment was greater than that of the original virus by amounts varying from 2 to 40 %. This variable increase may possibly be due to exposure of internal neuraminidase as a result of some virus disruption.

Purification and properties of the A 2 and B neuraminidases

The free neuraminidases released from A 2 and B viruses can readily be separated from residual virus and proteolytic enzyme by passage through Sephadex G 200 columns. Fig. 1 shows the result of passing 1 ml of pronase-treated A 2/ENG/67 virus through a 20 ml column of Sephadex G 200 equilibrated to phosphate buffered saline pH 6·0. The residual virus particles were excluded by the column.

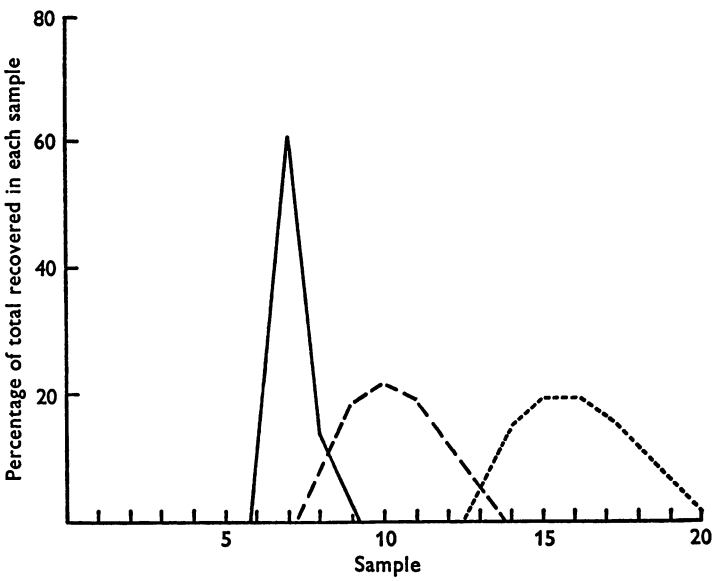


Fig. 1. Filtration of pronase-treated A 2/ENG/67 virus through Sephadex G 200. ———, Residual virus; – – – – –, neuraminidase; - - - - -, pronase.

Neuraminidase and pronase were retarded and well separated. Preparations purified in this way retained activity unchanged for up to 3 months when stored at 4° C., but activity was lost in preparations frozen and stored at −20° C.

Interaction of free neuraminidase and red blood cells. The neuraminidases of both A 2 and B viruses are not readily adsorbed by red cells. Even serial adsorption in the cold with high concentrations of red cells produces only slight reduction in activity. Nevertheless, the enzymes release as much or more N-acetyl neuraminic acid from red cells as does intact virus, and enzyme treated red cells are not agglutinated by virus indicating that haemagglutinin and neuraminidase react with the same substrate.

Morphology of the free neuraminidase. Laver & Valentine (1969) showed that free neuraminidase released from A 2 virus by treatment with sodium dodecyl sulphate had a characteristic morphology, consisting of an oblong head with a thin centrally attached tail. On removal of the detergent these subunits aggregated to give a 'cartwheel' appearance, the tails being linked at the centre while the heads were peripherally distributed (Pl. 1, Fig. 6). The overall diameter of the 'cartwheel' is 330 Å, while the individual heads are approximately 70 Å. in diameter.

The neuraminidase released by proteolytic enzyme treatment has a different appearance (Pl. 1, fig. 4). No polymerized structures are seen and the grid is covered by numerous small fragments giving an appearance that can be described as 'snow'. On close examination there seemed to be two types of fragment. One was a rod-shaped structure 20 Å. in diameter and with a variable length in the range of 70–80 Å. The other form was pleomorphic with dimensions of 50–70 Å. diameter. Several of this group could be resolved as cup-shaped structures resembling the heads of the detergent-produced neuraminidase. Indeed the two structures described here, the rod and the roughly isometric particle, could possibly represent the stem and head of the sub-units forming the structure in Pl. 1, fig. 5. Alternatively it is possible that the rod-shaped structures are incompletely digested haemagglutinin sub-units, and that the neuraminidase is released from the virus particle by proteolytic digestion of the stem.

DISCUSSION

Many studies have been made of the effects of proteolytic enzymes on influenza viruses. Sugg & Cleeland (1962) attempted to relate the trypsin resistance of virus haemagglutinins to the serological character, but in the present study no definite relation was found and it seems probable that variations in resistance are due more to differences in the ease with which the surface projections are split off by trypsin than to differences in susceptibility of the haemagglutinin protein to digestion.

The release of free neuraminidase from virus particles by treatment with proteolytic enzymes has been described by Mayron, Robert, Winzler & Rafaelson (1961) and Noll, Aoyagi & Orlando (1962) using trypsin, by Wilson & Rafaelson (1963) using chymotrypsin, by Reginster (1966*a*) using caseinase C, by Seto, Drzeniek & Rott (1966) and Reginster (1966*b*) using pronase, and by Kendal, Biddle & Belyavin (1968) using nagarse protease. By these methods free neuraminidases

have been obtained from influenza A 2 strains, influenza B, some strains of influenza A, and from fowl plague virus, but not from A 1 strains. Most of these results were obtained by using minimal periods of enzyme treatment.

In the present work prolonged treatment with high concentrations of enzyme has been used and the results indicate the existence of at least two and possibly three types of neuraminidase. The neuraminidases of the A 2 strains and the LEE strain of virus B are completely resistant to the action of proteolytic enzymes. They are also resistant to the action of agents disrupting the higher order structure of protein molecules such as sodium dodecyl sulphate (Laver, 1964) and high concentrations of urea (Hoyle, 1969*b*).

The neuraminidases of the A and A 1 strains which are destroyed by SDS and urea are also sensitive to destruction by proteolytic enzymes, but appear to be divisible into two groups, the A 1 strains and the DSP strain of virus A being highly susceptible while the PR 8 and SWINE strains show some degree of resistance.

The free neuraminidases released from the A2 and LEE strains by proteolytic enzymes do not have the same morphology as those released by detergent treatment. The tails and heads appear to be separated in the enzyme released product and no aggregates of the sub-units are found. It would seem probable that proteolytic enzymes release neuraminidase by a partial attack on the tails, possibly commencing at the ends. The heads appear to be more resistant to digestion.

REFERENCES

AMINOFF, D. (1961). Methods for the quantitative estimation of *N*-acetyl-neuraminic acid and their application to hydrolysates of sialomucoids. *Biochemical Journal* **81**, 384–92.

HOYLE, L. (1969*a*). The chemical reactions of the haemagglutinins and neuraminidases of different strains of influenza viruses. I. Effect of reagents reacting with aminoacids in the active centres. *Journal of Hygiene* **67**, 289–99.

HOYLE, L. (1969*b*). The chemical reactions of the haemagglutinins and neuraminidases of different strains of influenza viruses. II. Effects of reagents modifying the higher order structure of the protein molecule. *Journal of Hygiene* **67**, 301–10.

KENDAL, A. P.. BIDDLE, F. & BELYAVIN, G. (1968). Influenza virus neuraminidase and the viral surface. *Biochimica et biophysica acta* **165**, 419–31.

LAVER, W. G. (1964). Structural studies on the protein subunits from three strains of influenza virus. *Journal of Molecular Biology* **9**, 100–124.

LAVER, W. G. & VALENTINE, R. C. (1969). Morphology of the isolated haemagglutinin and neuraminidase subunits of influenza virus. *Virology* **38**, 105–19.

MAYRON, L. W., ROBERT, B., WINZLER, R. J. & RAFAELSON, M. E. (1961). Studies on the neuraminidase of influenza virus. I. Separation and some properties of the enzyme from Asian and PR 8 strains. *Archives of Biochemistry* **92**, 475–83.

NOLL, H., AOYAGI, T. & ORLANDO, J. (1962). The structural relationship of sialidase to the influenza virus surface. *Virology* **18**, 154–7.

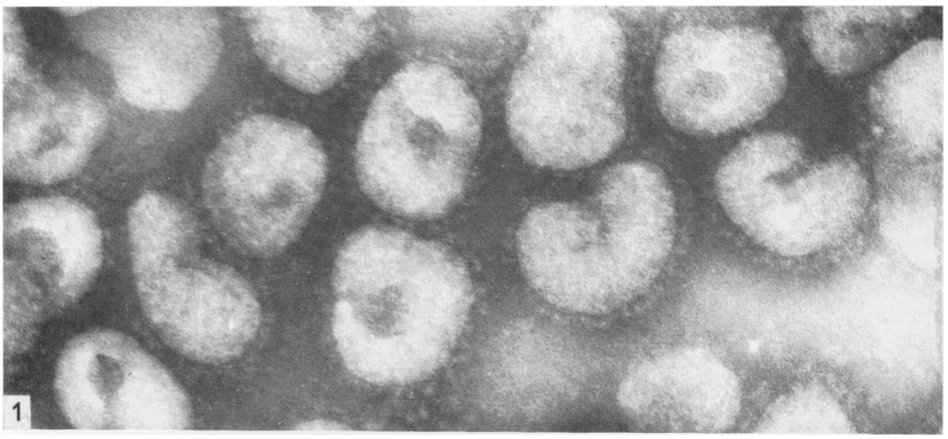
REGINSTER, M. (1966*a*). Release of influenza virus neuraminidase by caseinase C from *Streptomyces albus G. Journal of General Microbiology* **42**, 323–31.

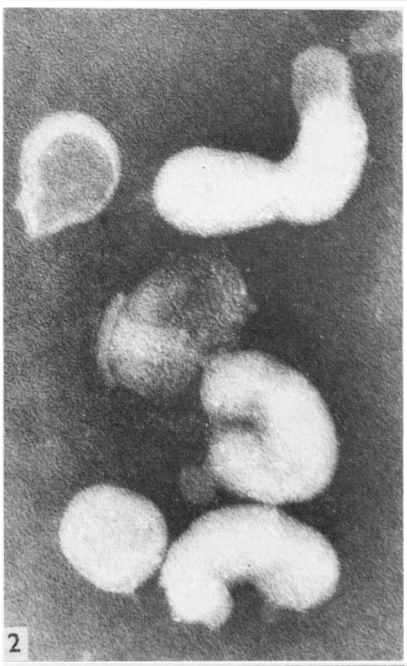
REGINSTER, M. (1966*b*). Effects of pronase on influenza virus. *Acta Virologica* **10**, 111–16.

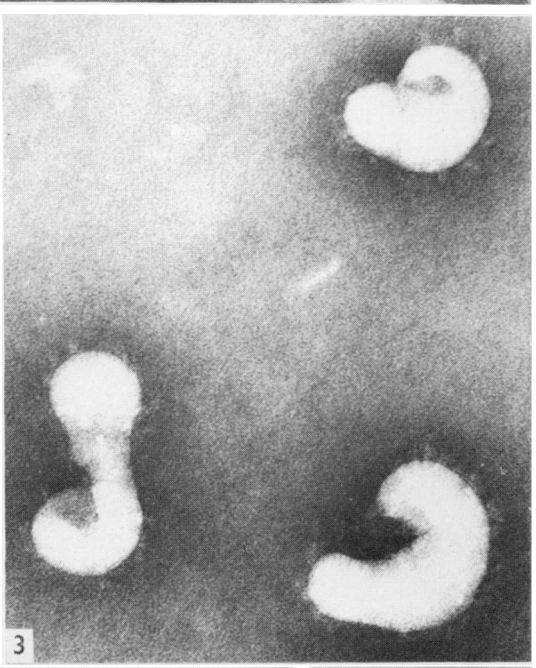
SETO, J. C., DRZENIEK, R. & ROTT, R. (1966). Isolation of a low molecular weight sialidase (neuraminidase) from influenza virus. *Biochimica et biophysica acta* **113**, 402–4.

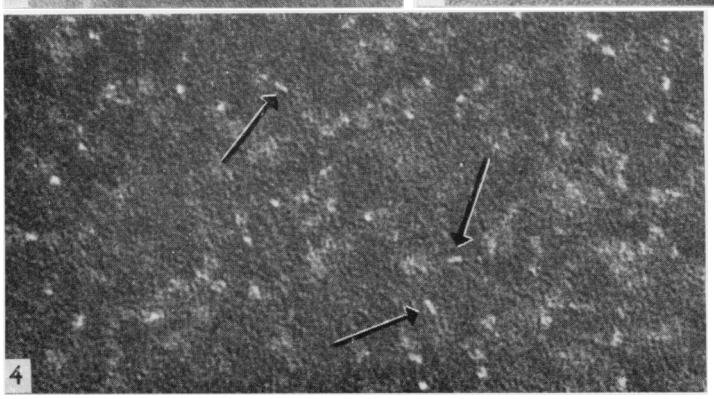
SUGG, J. Y. & CLEELAND R. (1962). Differences in trypsin susceptibilty among influenza viruses and relationship of the susceptibility to the antigenic type or subtype of the virus. *Journal of Immunology* **88**, 369–76.

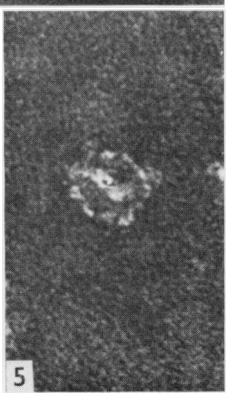
WILSON, V. W. & RAFAELSON, M. E. (1963). Isolation of neuraminidase from influenza virus. *Biochemical Preparations* **10**, 113–17.


1


2


3


4


5

EXPLANATION OF PLATE

Fig. 1. Untreated D.S.P. virus × 200,000. The particles are covered by projections 100 Å. in length.

Fig. 2. Pronase-treated D.S.P. virus × 200,000. The distinctive shape of the particles is still recognizable but there are no surface projections.

Fig. 3. Trypsin-treated D.S.P. virus × 200,000. The virus shows an appearance intermediate between figs 1 and 2. Projections are present on the surface but in fewer numbers than in untreated virus.

Fig. 4. Free neuraminidase from pronase-treated A 2/ENG/67 virus purified by passage through Sephadex G 200. × 300,000. Most of the particles appear pleomorphic and in the size range 50–70 Å, but a small number (arrows) have a distinct rod shape with a diameter of 20 Å. and variable length around 80 Å.

Fig. 5. Neuraminidase released from A 2/ENG/67 virus by treatment with the detergent Nonidet P 40. Here the individual units are polymerized into a wheel-shaped structure approximately 330 Å. in diameter. × 300,000.