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A COMPARATIVE STUDY OF INFLUENZA VIRUS NEURAMINIDASES, USING AUTOMATED TECHNIQUES

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SUMMARY

1. Automated techniques are described which may be used to investigate the kinetic behaviour and antigenic specificity of the enzyme neuraminidase (mucopolysaccharide *N*-acetylneuraminylhydrolase, EC 3.2.1.18).

2. Using these methods, the neuraminidases of a large number of strains of influenza virus were examined.

3. Combining the present results with other reported findings, it has proved possible to classify the enzymes of 23 virus strains into 5 kinetic and 10 antigenic groups.

4. This scheme provides further evidence that the molecular configurations forming the enzyme active site and the antigenic sites of influenza virus neuraminidases are different.

INTRODUCTION

Currently there is much interest in the antigenic nature and significance of the enzyme neuraminidase (mucopolysaccharide *N*-acetylneuraminylhydrolase, EC 3.2.1.18), which is incorporated into the structures of the myxoviruses. It has been shown that neuraminidase enzyme activity is associated with viral specific antigens that are distinct from the other viral components[1–12] and that neuraminidase is a minor surface component of the influenza viruses[12,13], probably contributing little to the stability of the viral envelope[14]. The serology of some representative influenza viruses has been investigated, and in some cases common antigenic determinants have been detected on the enzymes of different virus strains[7,10,11]. Although the heat stability of human influenza virus neuraminidases is apparently unrelated to their

Abbreviations: NANA, *N*-acetylneuraminic acid; 2–3 and 2–6 sialo-lactose, 2 → 3 and 2 → 6 isomers of *N*-acetylneuraminyllactose; EID_{50}; median egg infective dose.

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immunological specificity[10], other characteristic enzyme parameters have only been compared in a few cases[1,15]. Due to the large number of variants of influenza virus that have been isolated, it is clear that studies of their interrelationship can best be made by the use of automated procedures. In previous reports, the development of such an automated method suitable for assaying the enzyme activity of influenza virus neuraminidase has been described[16,17], and it seemed worthwhile to investigate the further applications of this method. The present report discusses the conditions whereby some kinetic aspects of neuraminidase enzyme activity, and the inhibition of neuraminidase enzyme activity, may conveniently be measured using automated techniques. Use of these methods has enabled a comparative study to be made of the neuraminidase activity of a large number of influenza virus strains of human and avian origin.

MATERIALS AND METHODS

Virus strains

The viruses used and their source are shown below.

A_2/Singapore/57, A_1/HAL/51, A_0/PR8/34, A_0/MEL/35, B/LEE/40, B/ROB/55 and B/England/13/65 (seeds maintained at University College Hospital Medical School); A_2/Hong Kong/68, A_2/Tokyo/67, A_1/Firenze/68, A_0/NWS/39, A/Turkey/Massachusetts/65, A/Turkey/England/63, A/Tern/South Africa/61, A/Chicken/Scotland/59, A/Dutch, A/Brescia, and virus 'N' (seeds supplied by Dr. H. G. Pereira, World Influenza Centre, Mill Hill, England); A/Rostock (seed supplied by Prof. R. Rott, University of Giessen, Germany); A/Turkey/Wilmot/63, A/Turkey/Wilmot/65 (seed supplied by Prof. G. Lang, University of Guelph, Ontario, Canada); A/Turkey/England/66 (seed supplied by Dr. C. C. Wannop, Houghton Poultry Research Station, England). Viruses were propagated in the allantoic cavity of 10–11-day-old chick embryos, in the presence of penicillin and streptomycin. Preparations referred to as 'spinco concentrates', were partially purified from infected allantoic fluid by one cycle of differential centrifugation.

Isolation of neuraminidase

Purified isolated neuraminidase was obtained from virus strains A_2/Singapore/57 and B/ROB/55 by proteolysis of purified virus concentrates, followed sequentially by a short and then a long period of rate zonal density gradient centrifugation. The neuraminidase obtained in this way from A_2/Singapore/57 has been shown to be homogeneous on analytical ultracentrifugation, with a sedimentation coefficient, s_{20}, of 7.8 S (ref. 13).

Neuraminidase substrates

(a) *N*-acetylneuraminyllactose (sialo-lactose) was isolated from bovine colostrum by dialysis and ion-exchange chromatography on a column of Dowex 1 X-2 resin. Sialic acid containing material eluted from the resin by pyridyl–acetate buffer was concentrated by freeze-drying, and finally purified by gel filtration through a column of Sephadex G-10 or G-15. The full procedure has been previously described[13]. Although no attempt was made to remove 2 → 6 isomer of *N*-acetylneuraminyllactose (2–6 sialo-lactose) from the product, this isomer has been shown to be present as only

8–16% of the total yield of sialo-lactose when bovine colostrum is taken as the source[18,19]. The specificity of influenza virus neuraminidase is believed to be almost exclusively for the predominant 2 → 3 isomer of *N*-acetylneuraminyllactose (2–3 sialo-lactose)[18,20].

(b) Human serum glycoprotein. This was kindly supplied as Cohn fraction IV-4, by Dr. D. S. Pepper, of the American National Red Cross, Washington, D.C., U.S.A., and contained 2% by weight acid-releasable sialic acid (25 mM H_2SO_4, 1 h, 80°).

Preparation of antiserum to human influenza virus strains

Purified virus was obtained from allantoic fluid by one cycle of differential centrifugation followed by rate zonal density gradient centrifugation as previously described[13]. 500–2500 haemagglutinating units of these preparations were then inoculated intravenously into rabbits, followed by a second inoculation of an equal virus dose after 21 days. Pre-inoculation (normal) sera and sera collected 3–5 days after the booster inoculation were used in inhibition tests. Ferret serum against A_0/PR8/34 was obtained by intranasal inoculation of 0.5 ml of a 1:10 dilution of infected allantoic fluid, and the ferret was bled after 15 days.

Preparation of antiserum to avian influenza virus strains

This was performed in association with Mr. W. H. Allen of the Central Veterinary Laboratory, New Haw, Weybridge, England. Chickens were maintained in isolation, and except for virus 'N', groups of 4–5-week-old chickens (20–30) were used for each virus strain. All inoculations were given intramuscularly, but due to the varying degrees of pathogenicity of the virus strains for chickens, several schedules were adopted. In the case of A/Turkey/Wilmot/63, A/Turkey/Wilmot/65 and A/Turkey/England/66 an initial virus dose was given ($10^{5.3}$ median egg infective dose (EID_{50})) followed 1 month later by a booster dose of infected allantoic fluid containing alhydrogel as adjuvant. Sera were collected after a further 2 weeks. Antiserum against virus 'N' was prepared by a single inoculation of infected allantoic fluid into a group of 12-week-old birds, and sera were collected after 3 weeks. In the case of the other, highly virulent strains, the chicks were preimmunized with 0.5 ml infected allantoic fluid treated with 1/1000 formaldehyde. After 14 days a single dose of live virus was given ($10^{5.3}$ EID_{50}), and sera were collected 2 weeks later when the majority of birds were sick or moribund.

Automated procedures for assaying neuraminidase activity

(a) General features. The apparatus used for determining free sialic acid resulting from the action of neuraminidase on substrate solution consists of standard Technicon autoanalyser modules, set up similarly to the manner shown previously (Fig. 1, ref. 17). Minor improvements have been made to the flow circuit, giving better resolution of sample peaks (Fig. 1). A modified sampler II module was used throughout, which simultaneously aspirates one solution from the sampler turn-table and one from a fixed stock solution, in the volume ratio of 5:1 (see ref. 13). The solutions used are described below, and a schematic diagram of the overall automated procedure is shown in Fig. 2.

(b) Investigation of the variation of neuraminidase activity with pH. For each experiment, a virus preparation in unbuffered physiological saline was sampled from

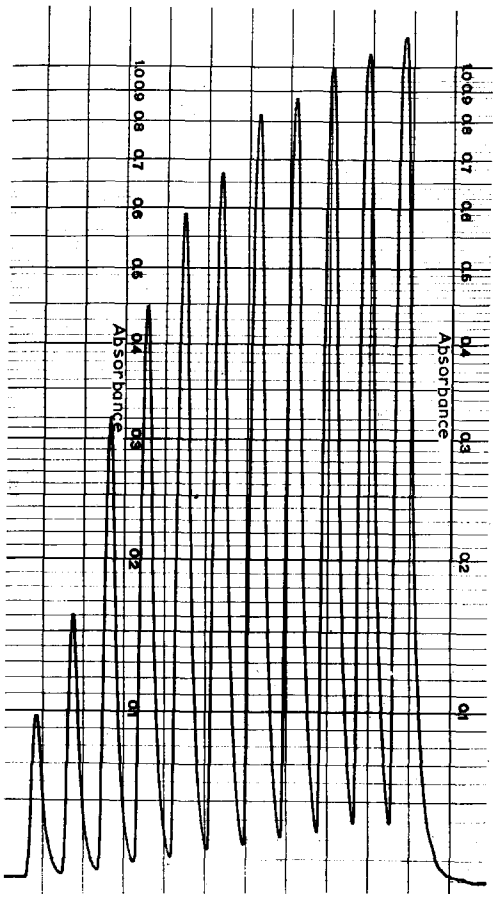


Fig. 1. Autoanalyser trace recording. Standard NANA solutions, from left to right: 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 μg/ml sampled at a rate of 20/h; sample: wash ratio 1:5.

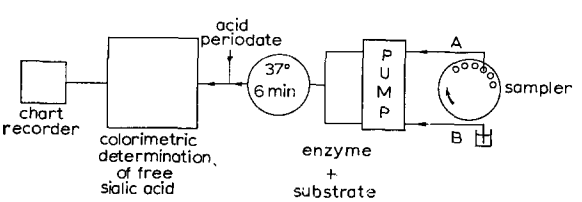


Fig. 2. Schematic representation of automated assay of neuraminidase activity. Enzyme and substrate preparations were aspirated through separate probes by means of a modified Technicon sampler II module[13]. Solutions sampled by the probes A and B are described in the text for each of the various applications of this automated procedure. Separate wash reservoirs were provided for the probes to prevent contamination of substrate by enzyme during the wash period between each sampling operation.

the fixed position, while a series of sialo-lactose substrate solutions were sampled sequentially from the turn-table. The substrate solutions were prepared in 0.1 M phosphate buffers, covering the pH range 5.5–7.3, and contained 180 μg releasable sialic acid per ml. Due to the dilution with enzyme solution, the final concentration of sialic acid in the incubation mixture was 150 μg/ml.

(c) Investigation of the variation of enzymic activity with substrate concentration. A similar arrangement was adopted to that of (b) above, but in this case the substrate solutions were prepared in 0.1 M phosphate buffer (pH 6.8), and the series of sialo-lactose solutions on the sampler tray contained concentrations of releasable sialic acid within the range 35–250 μg/ml. The actual concentration of substrate in the incubation medium was again calculated by applying the standard dilution factor.

(d) Investigation of the range of linearity of the enzyme assay. For this purpose, the position of the enzyme and substrate sample solutions was interchanged. Substrate solution of sialo-lactose was prepared in 0.1 M phosphate buffer (pH 6.8) and contained 900 μg releasable *N*-acetylneuraminic acid (NANA) per ml. As this was now sampled through the smaller input, a final concentration of 150 μg NANA per ml was obtained in the enzyme incubation coil. Virus suspensions within a suitable concentration range were prepared in the same buffer, and were sampled sequentially from the turn-table through the larger input. A unit of neuraminidase activity is defined for these conditions as the amount of enzyme releasing NANA equivalent to a concentration of 10^{-2} μmole/ml, as sampled from the turn-table.

(e) Determination of inhibition of neuraminidase activity by antisera, using sialo-lactose as enzyme substrate. Virus spinco concentrates were diluted in 0.1 M phosphate buffer (pH 6.8) to contain approx. 20–30 units of neuraminidase activity per ml. To aliquots of these virus samples, equal volumes of serum dilutions (prepared in the same buffer) were added, and the mixtures were then incubated at 37° for 40 min, before being transferred to sample cups and measuring residual neuraminidase activity as in (d) above. Controls of (i) virus, no serum; (ii) no virus, normal serum, were assayed in parallel. Preliminary experiments showed that the presence of excessive concentrations of serum in virus samples interfered with the functioning of the autoanalyser system. At serum dilutions of 1:50 or less, the reagent base line was decreased, and the peak height produced by standard solutions of NANA was considerably reduced. The former effect may have been due to reaction of serum components with the periodate reagent, and was overcome by using serum dilutions of 1:100 in the final virus samples. Reduction of peak height was attributed to the formation of a protein film on the inside walls of the glass tubing of the autoanalyser modules, allowing greater diffusion of solutes between the liquid segments flowing through the tubing. The result of this would be to spread the base of each sample peak over a greater time interval, with a concomitant decrease in the absorbance at the apex of each peak. Use of serum at a final dilution of 1:100 greatly reduced this effect, and after a period of about 4 h of continuous sampling (*i.e.*, 80 samples) under these conditions, the peak height of standard NANA solutions showed a reduction of about 25%. As the order of sampling when determining neuraminidase inhibition was such that each virus strain was tested against a number of antisera (about 4–8) before assaying the next virus strain, the change in absorbance between the first and last sample of any virus group due to nonspecific interference with the colorimetric reaction was about 2–3%.

At the completion of each days work, the glass coils were cleaned by pumping chromic acid through them for about 15 min, thereby restoring the peak height obtained with standard NANA solutions to their original value.

(f) Determination of inhibition of neuraminidase by antisera, using human serum glycoprotein as enzyme substrate. Cohn serum fraction IV-4 and virus samples (infected allantoic fluid) were added to 0.2 M acetate buffer (pH 6.0) to give a substrate concentration of 80 μg NANA per ml. 0.02% NaN_3 was included, to suppress microbial growth. Aliquots (1.0 ml) of the enzyme–substrate mixture (about 1–2 enzyme units) were put in conical base 2-ml technicon cups, and periodate-treated sera were added (0.01–0.1 ml). The enzyme–substrate–serum mixtures were then incubated at 37° for 17 h, after sealing and shaking the sample cups, and the enzyme reactions were stopped by the addition at room temperature of 0.5-ml volumes of 10% trichloroacetic acid. This step also precipitated large amounts of serum and substrate proteins, which were sedimented by mounting the sample cups in the mouths of standard glass centrifuge tubes, and centrifuging for 2–3 min in a bench centrifuge. Free sialic acid in the supernatant fluids in the cups was determined by the standard automated procedure[17]. Controls of sialic acid standard solutions were found to be stable to this trichloroacetic acid treatment, and incubation of control substrate solutions followed by trichloroacetic acid treatment gave a final absorbance reading corresponding to the release of 3% of the total sialic acid. As in this procedure most of the protein was removed from the samples before determining sialic acid, high concentrations of serum (up to 1:10 dilution) could be tested for anti-neuraminidase activity if necessary. The glass coils of the apparatus were cleaned periodically with chromic acid, as before, to maintain the sensitivity of detection of NANA.

RESULTS

Kinetics of neuraminidase activity

The pH optimum of neuraminidase activity against sialo-lactose substrate was found to lie within the range pH 6.4–7.0 for a number of strains tested, including samples of human A_0, A_2 and B viruses and avian influenza A viruses. Similar values have been reported by other workers[1,15,18,21,22]. It can be seen from Fig. 3 that the variation of enzymic activity with pH in the region of optimum activity was generally

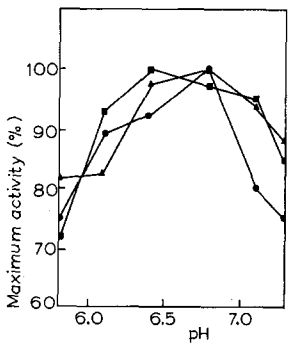
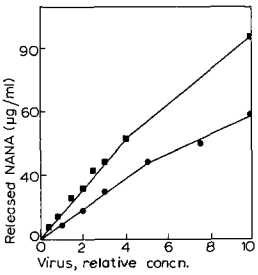


Fig. 3. Variation with pH of neuraminidase activity of some representative virus strains, against sialo-lactose substrate. ●—●, A_2/Singapore/57; ■—■, B/England/65; ▲—▲, A/Rostock.



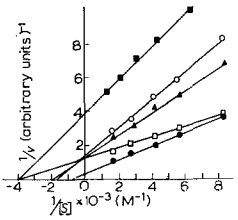


Fig. 4. Variation of neuraminidase activity with virus concentration, against sialo-lactose substrate. ●—●, A_2/Singapore/57; ■—■, B/Rob/55.

Fig. 5. Lineweaver–Burk plot showing the variation of neuraminidase activity with concentration of sialo-lactose substrate. ●—●, A_2/Singapore/57; ▲—▲, A_2/Tokyo/67; ■—■, B/England/65; ○—○, A_1/Firenze/68; □—□, A_0/PR8/34.

not very great, and accordingly a standard incubation medium of 0.1 M phosphate buffer (pH 6.8) was adopted for other experiments where sialo-lactose substrate was used. The greatest difference observed between the activity at this pH, and the activity at the true optimum pH for any enzyme was less than 15%. At pH 6.8, and under the conditions described in MATERIALS AND METHODS, the upper limit of linearity of the assay with respect to enzyme concentration was at a point where about 21% and 28% of the total available substrate had been consumed in the case of A_2/Singapore/57 and B/ROB/55 viruses, respectively (Fig. 4). The range of linearity therefore corresponded to 0–12 units of A_2 enzyme and 0–16 units of B enzyme. Thus, only minor differences in the enzymic activity of the neuraminidases of different virus strains were observed in the above experiments.

However, when the effect of substrate concentration on enzymic activity was investigated, significant differences were found to exist among the various strains of virus. Results were plotted by the method of LINEWEAVER AND BURK[23] (Fig. 5), and values for the Michaelis constant, K_m, of each neuraminidase were determined (Table I). It can be seen that with rare exceptions results were obtained with good reproducibility even in those cases where the amount of enzyme present lay outside the range of linearity previously determined for this assay at one fixed substrate concentration. No consistent variations were obtained using different batches of sialo-lactose. Values for the K_m of four of these viral enzymes have previously been reported, and no result differs by greater than a 4-fold factor from those found here[1,15,24–26]. Since different assay systems were used in these cases comparison with the present results may not be valid. However, all the values shown in Table I were obtained under standard conditions, and it is clear that certain groups of influenza virus neuraminidases have common enzymic characteristics, and may be classified according to their Michaelis constant measured in this system (Table IV). The K_m of neuraminidase isolated by proteolysis of virus was very similar to that of the native virus enzyme, as found by other workers[1,15], and the enzymes of three variants of A_2/Singapore/57 could not be distinguished kinetically. It is unlikely therefore that the different biological properties of these variants is a direct consequence of an unusual neuraminidase activity (also see ref. 22). Similarly, the enzyme of the neuro-

TABLE I

MICHAELIS CONSTANT OF HUMAN INFLUENZA A VIRUS, HUMAN INFLUENZA B VIRUS AND AVIAN INFLUENZA VIRUS NEURAMINIDASES FOR SIALO-LACTOSE SUBSTRATE

Sialo-lactose batches designated: ion-exchange extraction Expt. No./gel filtration purification Expt. No. (see MATERIALS AND METHODS for details). Mean K_m value is given in parentheses.

Expt. no.	*Virus strain*	*Total units of enzyme*	$K_m \times 10^4$ *(M)*	*Sialo-lactose Batch no.*
	Human influenza A virus			
1	A_2/Singapore/57	5	10	5
2		25	18	5
3		15	13	5
4		25	11	8/A
5		21	11	8/A
6		10	9	8/B
7		3	13	9/A
8	*	24	15	5
9	*	6	13	5
10	**	5	13 (13)	5
11	Isolated neuramini-	5	9.1	6/B
12	dase A_2/Singa-	5	10	6/B
13	pore/57	9	10 (9.7)	5
14	A_2/Tokyo/67	8	5.0	8/A
15		9	5.0	8/A
16		6	4.7 (4.9)	8/B
17	A_2/Hong Kong/68	23	5.9	8/B
18		19	5.9	8/B
19		16	6.2 (6.0)	9/A
20	A_1/HAL/51	6	5.3	8/A
21		10	5.8 (5.6)	9/A
22	A_1/Firenze/68	15	6.2	8/A
23		16	6.2 (6.2)	8/A
24	A_0/NWS/39	6	2.9	6/B
25		5	4.2	6/B
26		12	3.3 (3.5)	6/B
27	A_0/MEL/35	13	3.3	8/A
28	A_0/PR8/34	11	3.7	6/B
29		14	2.6	8/A
30		13	2.6 (3.0)	8/A
	Avian influenza virus			
44	A/Rostock	20	14	6/B
45		13	12	6/B
46		25	13	8/B
47		7	8.0	6/B
48		19	10 (11)	8/A
49	A/Brescia	3	7.7	6/B
50		7	8.9 (8.3)	8/A
51	A/Chicken/Scotland/	5	8.7	6/B
52	59	2	7.4 (8.1)	8/A
53	A/Tern/South	17	8.0	8/A
54	Africa/61	6	6.9 (7.5)	9/A
55	A/Turkey/England/63	8	8.7	8/B
56	A/Turkey/Wilmot/63	13	8.0	8/B
57		14	8.0 (8.0)	9/A
58	A/Turkey/England/66	23	8.0	8/A
59		14	8.0 (8.0)	9/A
60	A/Turkey/Wilmot/65	26	7.0	8/A
61		14	7.7	9/A
62		20	6.5 (7.1)	9/A
63	A/Dutch	17	18	8/B
64		16	25 (22)	8/A
65	Virus 'N'	17	18	8/B
66		16	20 (19)	8/A
67	A/Turkey/	26	4.8	8/B
68	Massachusetts/65	14	4.7	8/B
69		13	4.3	9/A
70		15	3.7 (4.4)	9/A

* γ-Inhibitor-resistant variant of A_2/Singapore/57 (ref. 37).
** Plaque-forming variant of A_2/Singapore/57 (ref. 38).

TABLE I *(continued)*

Expt. no.	*Virus strain*	*Total units of enzyme*	$K_m \times 10^4$ *(M)*	*Sialo-lactose Batch No.*
	Human influenza B virus			
31	B/LEE/40	10	3.2	6/B
32		5	2.4	6/B
33		8	3.3	6/B
34		9	2.2	8/A
35		6	2.1	8/B
36		5	2.5 (2.6)	9/A
37	B/England/65	10	2.6	6/B
38		10	2.6 (2.6)	6/B
39	B/ROB/55	4	2.6	5
40		4	2.3	8/A
41		29	4.0	5
42		7	2.7 (2.9)	6/B
43	Isolated neuramini-dase B/ROB/55	5	2.7	6/B

tropic virus A_0/NWS/39 had an identical substrate affinity to the closely related non-neurotropic viruses A_0/PR8/35 and A_0/MEL/35.

Immunological cross-reactions of influenza virus neuraminidase

The antigenic interrelationships of influenza virus neuraminidases were investigated by determining the inhibition of enzymic activity produced by specific antisera prepared against these viruses. In the case of the avian influenza viruses, measurements of neuraminidase activity were made using sialo-lactose as the enzyme substrate. Because the enzyme–substrate incubation conditions were carefully chosen so that the enzyme assay was linear with enzyme concentration, the inhibition of neuraminidase activity could be determined with sufficient accuracy using serum at one dilution only, that the presence of significant levels of antibody could be observed with good reproducibility. Sera were used at a dilution of 1:100 throughout, and the inhibition of enzymic activity by homologous sera was generally about 50% (Table II). Several strong cross-reactions were also observed, showing that 5 major antigenic variants of neuraminidase could be distinguished among these avian influenza viruses (Table IV). Although the enzymic activity of virus A/Turkey/Wilmot/63 was inhibited by four heterologous antisera, the amount of inhibition was very low in these cases. The total spectrum of cross-reactions were not reflected in the other, major, surface antigen (the haemagglutinin) of these viruses, whose overall serological reactions are the subject of a separate report[27].

However, the sensitivity of detection of anti-neuraminidase antibodies in a system where neuraminidase activity is assayed using sialo-lactose substrate is low,

TABLE II

INHIBITION OF AVIAN INFLUENZA VIRUS NEURAMINIDASE ACTIVITY BY CHICKEN CONVALESCENT SERA: SIALO-LACTOSE SUBSTRATE

Results expressed as percent inhibition of enzymic activity (compared to a control of enzyme and substrate, no serum), produced by sera at a dilution of 1:100. For full details see text. –, < 10%: +, 10–19%; ++, 20–29%; +++, 30–39%; ++++, 40–49%; +++++, ⩾ 50%; N.T., not tested. All results showing ⩾ 10% inhibition are the mean of 2–5 determinations, except for serum 'N' where only one value was obtained in each case

Virus strain	*Serum*									
	A/Rostock	*A/Brescia*	*A/Chicken/ Scotland/ 59*	*A/Tern/ South/ Africa/61*	*A/Turkey/ England/ 63*	*A/Turkey/ Wilmot/63*	*A/Turkey/ England/ 66*	*A/Turkey/ Wilmot/65*	*A/Dutch*	*Virus 'N'*
A/Rostock	++++	++++	+++++	–	–	N.T.	N.T.	–	–	–
A/Brescia	+++++	+++++	+++++	–	–	–	–	–	–	–
A/Chicken/Scotland/ 59	+++++	+++++	+++++	–	–	–	–	–	–	–
A/Tern/South Africa/ 61	–	–	–	++++	++++	–	–	–	–	–
A/Turkey/England/ 63	–	–	–	+++	++++	N.T.	N.T.	N.T.	–	–
A/Turkey/Wilmot/63	–	N.T.	++	N.T.	–	+++++	+	+	–	+
A/Turkey/England/ 66	–	N.T.	–	–	–	+	+++++	+++++	–	–
A/Turkey/Wilmot/65	–	N.T.	–	–	N.T.	–	+++++	+++++	–	+
A/Dutch	–	–	–	–	–	–	N.T.	–	+++++	+++
Virus 'N'	–	–	–	–	–	–	–	–	++	+

since steric hindrance by antibody molecules bound to the enzyme is not very great against this low molecular weight trisaccharide (see DISCUSSION). Accordingly, for the measurement of inhibition of human influenza virus neuraminidase by rabbit immune sera, a higher molecular weight substrate (serum glycoprotein) and a lower virus concentration were used to increase the sensitivity of the test, as described in MATERIALS AND METHODS. Under these conditions, normal rabbit sera at a dilution of 1:50 produced a nonspecific inhibition of 45–55% of the control enzymic activity. The viral enzymes were therefore tested against immune sera at this serum dilution, and in those cases where enzyme activity was inhibited to greater than 55%, an end point for 50% inhibition was obtained using three or four serum dilutions (Table III).

TABLE III

INHIBITION OF HUMAN INFLUENZA VIRUS NEURAMINIDASE ACTIVITY BY IMMUNE RABBIT SERA: SERUM GLYCOPROTEIN SUBSTRATE

Virus strain	*Serum*							
	*A_0/PR8/34**	*A_1/HAL/51*	*A_2/Singapore/57*	*A_2/Tokyo/67*	*A_2/Hong Kong/68*	*B/LEE/40*	*B/ROB/55*	*B/England/65*
A_0/PR8/34	650***	<	<	<	<	<	<	Not tested
A_1/HAL/51	<****	500	<	<	<	<	<	Not tested
A_2/Singapore/57	<	<	7500	200	100	<	<	Not tested
A_2/Tokyo/67	<	<	475	2500	750	<	<	Not tested
A_2/Hong Kong/68	<	<	75	2700	2800	<	<	Not tested
B/LEE/40	<	<	<	<	<	70	80	<
B/ROB/55	<	<	<	<	<	<	575	175
B/England/65	<	<	<	<	<	<	95	95

* Ferret serum.

** Titres expressed as the reciprocal of serum dilution producing 50% inhibition of enzymic activity compared to a control of enzyme and substrate, no serum; for full details see text.

*** < Inhibition of activity by serum at 1:50 dilution less than 55%.

Although the inhibition of some of the human influenza B viruses was very low, specific antibody was clearly detected in these cases when the sera were tested at a dilution of 1:50. At this dilution, even low concentrations of specific antibody inhibited more than 80% of the enzymic activity. Compounding all these results with those published by other authors[10,11,15,28] enables a classification to be made of the antigenic relationships of those viral neuraminidases whose kinetic relationships have been investigated here. Although the scheme shown in Table IV is to some extent arbitrary, for the purpose of discussion the groupings shown seem quite reasonable in view of all the experimental results obtained. The possibility that some of the avian virus neuraminidases other than A/Turkey/Massachusetts/65 are antigenically related to human virus neuraminidases remains to be investigated.

DISCUSSION

The automated procedure recently developed in this laboratory for determining neuraminidase activity is fundamentally similar to that used for many other enzyme

TABLE IV

CLASSIFICATION OF INFLUENZA VIRUS NEURAMINIDASES ON THE BASIS OF KINETIC AND ANTIGENIC CHARACTERISTICS

Kinetic groupings: P Q R S T
Mean $K_m \times 10^4$ (M): 2.6–3.5 4.4–6.2 7.1–8.3 11–13 19–22
References to antigenic groupings: (a) ref. 10, (b) present report, (c) ref. 7, (d) ref. 28, (e) ref. 15, (f) ref. 11.

Virus strain	*Neuraminidase kinetic grouping*	*Neuraminidase major antigenic grouping*
A_0/PR8/34	P	I (a), (b)
A_0/MEL/35	P	I (a)
A_0/NWS/39	P	Not Tested (a)
A_1/HAL/51	Q	II (b)
A_1/Firenze/68	Q	Not tested
A_2/Singapore/57	S	III (b), (c)
A/Turkey/Massachusetts/65	Q	III (c)
A_2/Tokyo/67	Q	IV (b), (d)
A_2/Hong Kong/68	Q	IV (b), (d)
B/LEE/40	P	V (a), (b)
B/ROB/55	P	V (b),
B/England/65	P	V (b)
A/Rostock	S	VI (b), (e)
A/Brescia	R	VI (b)
A/Chicken/Scotland/59	R	VI (b)
A/Tern/South Africa/61	R	VII (b)
A/Turkey/England/63	R	VII (b)
A/Turkey/Wilmot/63	R	VIII (b), (f)
A/Equine 2/Miami/63	Not tested	VIII (f)
A/Turkey/England/66	R	IX (b)
A/Turkey/Wilmot/65	R	IX (b)
A/Dutch	T	X (b)
Virus 'N'	T	X (b)

assays. This type of system may be advantageously used for investigating the behaviour of an enzyme under various incubation conditions, since it is only necessary to sample a constant concentration of enzyme solution, while simultaneously sampling a varying concentration of some other factor, such as substrate, and thereby the enzyme–substrate Michaelis constant may be determined. In the present experiments, the concentration gradient of substrate was produced by aspirating discrete solutions sequentially from the sampler turn-table. Although this procedure has been adopted for other enzyme systems[29], an alternative possibility is to sample continuously the concentration gradient produced by a suitable mixing device[30,31]. However, this latter method requires that the concentration gradient must be carefully calibrated, and also entails a high consumption of material. Apart from these practical considerations, there may also be valid theoretical objections to the use of continuous concen-

tration gradients when determining enzymic activity in a Technicon autoanalyser[32]. It would seem that for enzyme studies in general, the use of 2 (or more) synchronized sampler modules would permit the greatest flexibility of operation for automated procedures, providing facilities for multiple enzyme analysis[29,33] or other complex investigations involving variations in the conditions of enzymic incubation, with none of the disadvantages of sampling continuous concentration gradients.

Application of the automated procedure for measuring neuraminidase activity after neutralization with antibodies posed special problems, in particular those resulting from the inability of the autoanalyser to function correctly in the presence of high concentrations of serum protein. This could be most satisfactorily overcome by precipitating excess protein with trichloroacetic acid before determining sialic acid, with the added advantage that a high molecular weight glycoprotein could be used as the neuraminidase substrate.

The results obtained when the influenza virus neuraminidases were examined by these automated procedures prove to be of considerable interest. Immunologically, the total information available indicates that several groups of two or three enzymes may be identified, each group being characterized by a common major antigenic determinant. However, although generally each group of serologically related enzymes is also associated with a common enzymic property (or kinetic determinant) as shown by the K_m values, exceptions to this are found in the case of A/Rostock, and A/Turkey/Massachusetts/65 (see Table V). Further, it can be seen that serologically different groups of enzymes may have a similar kinetic determinant. Apparently, therefore, the formation of a particular entigenic grouping on a molecule of neuraminidase does not necessarily dictate the nature of the active site of that enzyme.

This conclusion is in agreement with the view that the inhibition of enzyme activity by antibody neutralization is due to steric hindrance of the active site[34,35]. Thus, the antibody molecules are probably directed not at the active site of the enzyme, but at sites some distance away. This view has been confirmed by the demonstration of a relationship between the size of substrate molecules and the effectiveness of inhibition of neuraminidase activity by a specific antiserum[1,36]. It is therefore quite possible to consider that the active sites of human influenza B neuraminidases are identical (or very similar) to those of the human A_0 enzymes, although the 2 are immunologically distinct. As the B virus strains were first isolated during the period of human A_0 influenza infections, this may represent a link between the 2 types of virus. Similarly, the recently isolated human virus strains A_2/Tokyo/67 and A_2/Hong Kong/68 may have neuraminidases whose active sites are structurally identical to those of the human A_1 viruses, which have not caused epidemics since 1956. There is therefore some evidence for the recurrence of structural features of the enzymes of influenza viruses, after a number of years during which they have not been detected in epidemic virus strains. Similar considerations may apply to the avian virus enzymes.

As yet it is not possible to determine what changes in the molecular structure of the viral enzymes causes these variations in their observed activity, and it is difficult to interpret what significance the K_m values may have to the biological role of neuraminidase in virus infections. The rather limited range of K_m values found for these viral enzymes may be the result of natural selection acting on the possible range of variation. Alternatively, it may be that fundamental considerations govern-

ing the arrangement of amino acids to form specific stereochemical binding and hydrolytic sites for sialo-glycosides severely limit the possible variations in conformation of such enzyme sites, and thus also limit their range in activity. The molecular weight of influenza virus neuraminidase, about 220 000 (ref. 13), is such that the molecules may well consist of several polypeptide chains. If this were so, it is possible that a genetically controlled variation in one chain could cause an alteration at the enzyme active site without changing the overall antigenic characteristics of the molecule, or *vice versa*. This situation has many interesting possibilities as a model for explaining in part the epidemic behaviour of the influenza viruses. Therefore the neuraminidases of recombinant virus strains might merit investigation of both their kinetic and antigenic nature, in relation to parent strains, to see whether these properties are genetically segregatable.

Although the K_m value determined for each viral enzyme is a constant, characteristic for that strain, it is not possible to equate enzymic activity with enzyme concentration when comparing enzymes in different kinetic groups, due to their varying response to changes in substrate concentration. When investigating the neutralization of enzymic activity by antibody, therefore, it may not be possible to standardize both the weight dose and activity dose of enzymes in different kinetic groups when enzyme assays are performed at one standard substrate concentration. Values for the neuraminidase inhibition titres of different antisera should be compared with this reservation in mind.

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