Inactivation of Influenza Virus by Caseinase C from *Streptomyces albus* G Culture-filtrate

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SUMMARY

After incubation for 1 hr at 37° with caseinase C (a purified fraction of actinomycetin), the neuraminidase activity of concentrated purified influenza virus PR 8 suspension was unchanged, whereas its infectivity and haemagglutinating activity were considerably decreased. After 4 hr, infectivity and haemagglutinating activity were still more decreased and most of the neuraminidase activity was destroyed. The ability to fix complement in the presence of specific antibodies was slightly decreased, whereas the ability to neutralize haemagglutination-inhibiting antibodies was not affected.

Influenza virus PR 8 suspension treated with enzyme caseinase C contained material sedimentable by centrifugation for 1 hr at 31,000 *g* and material which remained in the supernatant fluid under these conditions. Both materials fixed complement in the presence of control PR 8 virus antiserum. The ability of control PR 8 virus suspension to neutralize haemagglutination-inhibiting antibodies and its ability to react with strain-specific complement-fixing antibodies were related to material sedimentable by centrifugation for 1 hr at 31,000 *g*. Treatment of PR 8 virus suspension by caseinase C destroyed its ability to produce antibody-fixing complement in the presence of control influenza virus PR 8. But this treatment did not suppress ability to produce specific haemagglutination-inhibiting antibodies. Following this treatment of PR 8 virus a new antigenic activity was shown: the antiserum to enzyme-treated virus not only fixed complement in the presence of enzyme-treated PR 8 virus, but also in the presence of enzyme-treated Asian virus. Material which fixed complement in the presence of antiserum to enzyme-treated virus was sedimentable from enzyme-treated PR 8 virus suspension by centrifugation at 31,000 *g*.

INTRODUCTION

Malchair (1958) reported that *Streptomyces albus* strain G culture filtrates obtained under suitable conditions, or actinomycetin, impaired the haemagglutinating activity and the infectivity of influenza virus by an enzymic process. In the present paper the action of a purified fraction of actinomycetin, caseinase C (Ghuysen, Leyh-Bouille & Dierickx, 1962; Dierickx & Ghuysen, 1962) on concentrated and purified influenza virus PR 8 is examined, its effects on the following viral properties being compared: haemagglutination, infectivity, neuraminidase activity, ability to react with specific antibody, antigenicity.

METHODS

Viruses. Influenza A virus strain PR8 was purified and concentrated by one adsorption-elution cycle followed by centrifugation. Packed fowl red cells (2%, v/v) were added to infected allantoic fluid obtained after three passages of lyophilized virus. Agglutinated red cells with virus adsorbed were washed twice with cold saline and resuspended in a volume of saline equal to one-fifteenth of the original volume of allantoic fluid. After incubation for 4 hr at 37°, the suspension was clarified by centrifugation at 1500 g for 15 min.; the eluted virus was then sedimented by centrifugation at 26,000 g for 60 min. The pellet was resuspended in a volume of distilled water equal to one-hundredth of the original volume of allantoic fluid. The viral suspension, after removal of large particles by centrifugation at 5000 g for 10 min., was distributed in sealed vessels which were stored in solid CO₂, or were frozen in solid CO₂ and afterwards kept at -70°. This preparation is referred to as concentrated purified PR8 virus. A stock of a Belgian strain A2 virus isolated in 1963 was similarly prepared.

Caseinase C preparation. The caseinase C used in this work was obtained from Dr J. M. Ghuyssen and Mr L. Dierickx; its purification will be reported elsewhere. By its action, casein becomes progressively less, and finally not at all, precipitable by trichloroacetic acid. The presence in the digest of at least four compounds highly reactive to ninhydrin indicates the proteolytic activity of this enzyme preparation (Mr L. Dierickx, personal communication). The solution of caseinase C used had the same caseinolytic activity as a solution containing 150 µg. crystalline trypsin/ml. Two different stocks were used which behaved identically.

Enzyme treatment of virus. Virus, caseinase C preparation and phosphate buffer (pH 7, µ 0.1) were mixed in the following proportions by vol.: concentrated purified virus, 31; caseinase C solution, 5; buffer, 4. Virus and enzyme were thus finally in µ 0.01 phosphate buffer at pH 7. The mixtures were incubated at 37°.

Measurement of virus haemagglutinating activity. From a 10⁻² dilution of virus suspension, several series of twofold dilutions were made in 0.5 ml. saline, on china or Perspex cup trays. To each cup was then added 0.5 ml. 0.5% (v/v) fowl red cell suspension. The haemagglutinating titre was calculated as the arithmetic mean of the reciprocal of the highest final dilution which gave detectable, although incomplete, haemagglutination in replicate series (most often four) after overnight incubation at room temperature. The amount of virus giving partial haemagglutination was taken as one haemagglutinating unit. When incomplete haemagglutination was not observed in a series, the titre was taken as the arithmetic mean between the highest dilution to give complete haemagglutination and the one immediately following which gave a negative result.

Measurement of virus infectivity. The technique with fragments of chorioallantoic membranes on the shell was used (Fazekas de St Groth & White, 1958). Sixteen twofold dilutions, the range of which included the ID₅₀ dose were inoculated, with 10 cups/dilution. Two Perspex trays were put side by side on each of six aluminium shelves supported by a stainless steel frame and spaced at intervals of 2.5 cm. The ID₅₀ dose was estimated graphically on logarithmic probit paper (Boyd, 1956).

Measurement of neuraminidase activity. The substrate was human urinary mucoprotein (Tamm & Horsfall, 1950). Free N-acetylneuraminic acid was esti-

mated by the sodium iodate + thiobarbiturate assay of Warren (1959; readings taken at 549 m μ). Each result was the average of four assays.

Measurement of the ability of virus to react with specific antibodies

Antiserum. Antiserum to influenza virus PR8 was obtained from a rabbit after intramuscular injections of virus purified by a single adsorption-elution cycle, the saline containing the eluted virus being cleared of red cells by centrifugation at 1500 *g* for 15 min., and immediately distributed in screw-cap bottles and stored at -25° . The rabbit was given 1.0 ml. injections of this suspension, the first five twice a week, the sixth 32 days after the fifth, and the seventh 24 days later. The rabbit was bled 7 days after the last injection. The serum was stored at -25° in screw-cap bottles.

Complement-fixation test. A micromethod in tubes was used (Sohier, Peillard, Gineste & Freydier, 1956) with complement constant at 2 units. Twofold dilutions of antiserum were tested in the presence of twofold dilutions of viral suspension. Fifty % haemolysis was taken as the end-point. Anticomplementary activity of rabbit serum was treated by diluting 1/10 in veronal buffer (Sohier *et al.* 1956) and heating at 56° for 30 min. An equal volume of guinea-pig serum diluted 1/2 in veronal buffer was added. The mixture was kept at 37° for 60 min. and afterwards heated at 56° for 30 min.

Absorption of haemagglutination-inhibiting antibodies. To titrate the ability of a virus suspension to absorb antibody from a given dilution of antiserum and to measure the limiting concentrations of virus which neutralized various concentrations of antiserum, the following two tests were used. (1) Twofold dilutions of virus were added to equal volumes of antiserum diluted 1/80; 60 min. later, twofold dilutions were prepared from these mixtures, using 0.25 ml. volumes; to each dilution was added four haemagglutinating PR8 units in 0.25 ml. and 60 min. later, 0.5 ml. red cell suspension. (2) Twofold dilutions of antiserum and of virus suspension were prepared. One-tenth ml. of each antiserum dilution was added to 0.1 ml. of each virus dilution. The mixtures were left for 60 min. before adding four haemagglutinating units of PR8 in 0.2 ml. and 60 min. later, 0.4 ml. of red cell suspension. Saline was used as diluent. Antisera were previously heated at 56° for 30 min. The four haemagglutinating PR8 units were in the form of diluted infected allantoic fluid. A 0.5 % (v/v) fowl red cell suspension was used. The tests were made at room temperature and read as soon as red cell sedimentation was complete in the diluent.

Antigenicity was tested by comparing two sera.

(a) *Sera.* Two guinea-pigs received six intraperitoneal injections of enzyme-treated or control PR8 virus suspension, respectively. The first five injections were done twice a week, the sixth 24 days after the fifth. The two animals were bled 4 days after the last injection. The stock of concentrated purified virus prepared for this purpose was obtained according to the technique described above, but by using two adsorption-elution cycles and a final centrifugation at 1500 *g* for 15 min. On each inoculation day 0.75 ml. of virus suspension was thawed. One half was incubated for 4 hr at 37° in the presence of caseinase C, the other half kept at 37° in phosphate buffer (μ 0.01; pH 7) for the same time. The effect of the enzyme was checked by a haemagglutination test. Control virus suspension diluted 1/10 in saline containing 1000 units penicillin G and 1000 μ g. streptomycin sulphate/ml.

was inoculated into the guinea-pig for control antiserum. To the enzyme-treated virus suspension diluted 1/10 in saline containing antibiotics, 20 % (v/v) of packed fowl red cells were added; after 15 min. at room temperature, this mixture was centrifuged for 15 min. at 1500 *g*. The supernatant fluid obtained after four such adsorptions was inoculated into the guinea-pig to produce antisera to enzyme-treated virus.

(b) *Inhibition of haemagglutination.* Twofold dilution series of sera previously treated by periodate to destroy non-specific inhibitors were prepared on china or Perspex cup trays. To 0.25 ml. of each serum dilution, four haemagglutinating units of virus in 0.25 ml. were added. After 60 min. at room temperature, 0.5 ml. of 0.5 % (v/v) fowl red cells were added. Readings were made as soon as red cell sedimentation was complete in the diluent (saline), the end-point being defined as the highest dilution at which no haemagglutination was observed.

RESULTS

Haemagglutinating activity

The haemagglutinating activity of concentrated purified influenza PR8 virus remained unchanged after incubation for 4 hr at 37°, in phosphate buffer (pH 7; ionic strength μ 0.01). In the presence of caseinase C, 90–99 % of the haemagglutinating activity was destroyed (Table 1). About 55 % of the destruction observed after 4 hr at 37° took place during the first hour of incubation (Table 3).

Table 1. *Haemagglutinating activity of concentrated purified PR8 virus (0.01 μ phosphate buffer pH 7), after 4 hr of incubation at 4° without caseinase C and at 37° with and without caseinase C*

Exp. no.	Haemagglutinating titre after incubation			
	Without caseinase C at		With caseinase C at 37°	
	4°	37°	Absolute	%
1	51,200	51,200	4,800	9.4
2	83,200	89,600	800	0.9
3	89,600	70,400	1,600	2.3
4	153,600	153,600	3,200	2.1
5	76,800	76,800	400	0.5
6	—	153,600	1,900	1.2

Infectivity

Incubation for 4 hr at 37° at pH 7 and μ 0.01 did not decrease the infectivity of concentrated purified PR8 virus. After the same length of time at 37° in the presence of caseinase C, the titre of the viral preparation was decreased to between 10^{-3} and 10^{-4} of its former value (Table 2).

Neuraminidase activity

The neuraminidase activity of PR8 virus was estimated on the basis of the amount of *N*-acetylneuraminic acid liberated, after incubation for 4 hr at 37°, by 1 part of viral suspension acting upon 9 parts of the mucoprotein solution (water containing penicillin 100 units/ml. and streptomycin 100 μ g./ml.). This system contained 550 μ g./ml. *N*-acetylneuraminic acid liberable by acid hydrolysis (0.1 *N*-H₂SO₄; 1 hr

at 80°). Under the conditions of our experiments, the relationship between the concentration of the viral suspension and the amount of free *N*-acetylneuraminic acid was given by the equation:

$$\log y = a \log x,$$

where *a* is a constant, *x* the concentration of the virus in percentage of the undiluted control, and *y*, the amount of free *N*-acetylneuraminic acid as % of the *N*-acetylneuraminic acid liberated after incubation for 24 hr in the presence of undiluted control virus suspension (about half of the *N*-acetylneuraminic acid freed

Table 2. *Infectivity of concentrated purified PR8 virus after 4 hr of incubation (phosphate buffer, pH 7, μ 0.01) at 4° without caseinase C and at 37° with and without caseinase C*

Expt. no.	Infectivity after incubation					
	Without caseinase C at					With caseinase C at
	4°		37°		37°	
	$\log_{10}$ ID 50 in 1 ml.	Standard error*	$\log_{10}$ ID 50 in 1 ml.	Standard error*	$\log_{10}$ ID 50 in 1 ml.	Standard error*
1†	7.74	0.12	7.74	0.14	4.77	0.06
2†	7.85	0.09	7.92	0.08	3.64	0.17
3	8.12	0.15	8.12	0.13	—	—
4	—	—	7.44	0.14	4.11	0.12

* Finney (1947).

† Sample of the virus suspensions used respectively for Expts. no. 1 and 2 of Table 1.

Table 3. *Haemagglutinating activity, infectivity and neuraminidase activity of concentrated purified PR8 after incubation at 37° in the presence of caseinase C for different periods of time*

Length of incubation (hr)	Activity in percentage of control										
	Haemagglutination expt.					Infectivity expt.		Neuraminidase expt.			
	1	2	3	4	5	1	3	1	3	4	5
1	41	47	37	66	66	5.0	0.1	101	147	120	116
2	33	23	25	33	25	—	—	32	68	81	80
4	1	2	3	1	1	—	—	3	34	10	9
6	—	—	—	—	—	—	—	—	—	3	3

by acid hydrolysis). By this technique it was found that the neuraminidase activity of various dilutions of concentrated purified virus at pH 7, μ 0.01, was the same after incubation for 4 hr at 4° as at 37° (Fig. 1). After 4 hr at 37° in the presence of caseinase C the virus suspension liberated from 0 to 34 % of the amount of *N*-acetylneuraminic acid liberated by the control, which means that 75–97 % of the original neuraminidase activity had disappeared. After incubation for 1 hr the neuraminidase activity remained unchanged (Table 3).

Ability to react with specific antibodies

In the presence of rabbit antiserum, concentrated purified PR8 virus suspension previously incubated for 4 hr at 37° (pH 7, μ 0.01) fixed complement after overnight incubation at 4°. After an identical pre-incubation, but in the presence of caseinase

Fig. 1

Fig. 2

Fig. 1. Neuraminidase activity of dilutions of concentrated purified PR 8 after 4 hr incubation at 37° or 4°, pH 7, μ 0.01. Ordinates: $\log y$, where y is the amount of *N*-acetylneuraminic acid liberated after 4 hr incubation at 37° in % of the *N*-acetylneuraminic acid liberated by the undiluted virus suspension after 24 hr incubation at 37°. Abscissae: $\log x$, where x is the concentration of the virus in % of the undiluted virus suspension. Expt. no. 1: 37°, ●; 4°, ○. Expt. no. 2: 37°, ▲; 4°, △.

Fig. 2 Neutralization by haemagglutination-inhibiting activity of anti-PR 8 rabbit serum by various dilutions of PR 8 virus suspension incubated at 37°, μ 0.01, pH 7 with or without caseinase C, further adsorbed by 20% fowl red cells or not. Shadowed band: haemagglutination in the presence of diluent alone. White bands: haemagglutination in the presence of various dilutions of concentrated purified PR 8 incubated at 37°, μ 0.01, pH 7. Pattern obtained by using control virus, virus treated by caseinase C or virus treated by caseinase C and further adsorbed by 20 % fowl red cells. Black bands: haemagglutination in the presence of various dilutions of concentrate purified PR 8 after 4 hr incubation at 37°, μ 0.01, pH 7 and adsorption on 20 % fowl red cell

Table 4. Complement fixation, after overnight incubation at 4°, by concentrated purified PR8, untreated or treated by the caseinase C in the presence of anti-PR8 rabbit serum

Virus	Virus dilution	Complement fixation in the presence of antiserum diluted			
		1/20	1/40	1/80	1/160
Control	1/2	+	+	+	0
	1/4	+	+	0	0
	1/8	+	+	0	0
	1/16	+	+	0	0
	1/32	+	0	0	0
	1/64	0	0	0	0
After enzymic treatment	1/2	+	+	0	0
	1/4	+	+	0	0
	1/8	+	+	0	0
	1/16	+	0	0	0
	1/32	0	0	0	0
	1/64	0	0	0	0

Average of the results obtained with samples of the virus suspensions used for Expts. nos. 1 and 2 of Tables 1 and 2.

C, the ability of the virus to fix complement in the presence of rabbit antiserum was slightly decreased, i.e. in the presence of serum diluted 1/20, the minimum active virus concentration was twice as high for treated virus as for the control, and conversely the serum titre measured in the presence of control virus diluted 1/2 was twice as high as that measured in the presence of the same dilution of treated virus (Table 4). This rabbit antiserum diluted 1/20 did not fix complement in the presence of standard soluble A antigen extracted from allantoic cells infected with PR8 virus.

After incubation for 4 hr at 37° in the presence of a caseinase C preparation, the ability of concentrated purified PR8 virus suspension to bind haemagglutination-inhibiting antibodies was not modified. By measuring non-absorbed antibodies, it was found that, when added to an equal volume of antiserum diluted 1/80, treated or untreated virus, diluted 1/200, caused the antiserum to lose 75% of its ability to inhibit haemagglutination. Virus preparations (treated or untreated) diluted 1/400 had no effect on the haemagglutination-inhibiting titre of the antiserum diluted 1/80. On the other hand, neutralization of haemagglutination-inhibiting antibodies was obtained by mixing various amounts of virus with various amounts of antiserum. The same limiting concentrations of treated and untreated virus suspensions neutralized each tested concentration (Fig. 2).

Ability of virus to adsorb on fowl red cells

Concentrated purified PR8 virus suspension previously incubated at 37° for 4 hr in the presence of caseinase C preparation was diluted 1/10 in saline and 20% (v/v) of packed fowl red cells added. After 15 min. at room temperature, this mixture was centrifuged for 15 min. at 1500 g. The supernatant fluid was tested for ability to absorb haemagglutination-inhibiting antibodies. The same limiting concentrations of both enzyme-treated virus suspension and adsorbed enzyme-treated virus suspension neutralized each tested concentration of antiserum (Fig. 2). Adsorption of control virus suspension by fowl red cells under the same conditions strongly decreased the ability of this suspension to neutralize haemagglutination-inhibiting antibodies (Fig. 2).

Antigenicity

Antiserum to control (untreated) virus and antiserum to enzyme-treated virus were examined for haemagglutination-inhibiting antibody and for antibody which fixed complement in the presence of standard soluble antigen and in the presence of enzyme-treated (*E* suspension: concentrated purified virus incubated at 37° for 4 hr in the presence of caseinase C) and control (*C* suspension: concentrated purified virus incubated for 4 hr at 37°); phosphate buffer pH 7, μ 0.01, virus suspensions of both influenza PR8 and Asian influenza (Table 5).

Antiserum to control virus and antiserum to enzyme-treated virus inhibited four haemagglutinating units of PR8 virus to 1/5120 and 1/320, respectively.

Antiserum to control virus fixed complement in the presence of enzyme-treated and control PR8 virus suspensions. The titre of antiserum to untreated virus in the presence of one unit of enzyme-treated PR8 virus antigen (*E* suspension diluted 1/2) was 1/80. Its titre in the presence of one unit of homologous antigen (*C* suspension diluted 1/8) was 1/320.

Table 5. Complement fixation, after overnight incubation at 4° by various dilutions of concentration purified PR8 and Asian viruses, untreated or treated by caseinase C, in the presence of untreated PR8 virus guinea-pig antiserum or treated PR8 virus guinea-pig antiserum

Virus	Virus diluted	Complement fixation in the presence of antiserum (guinea-pig)															
		Control PR 8 antiserum diluted								Enzyme treated PR 8 antiserum diluted							
		1/10	1/20	1/40	1/80	1/160	1/320	1/640		1/10	1/20	1/40	1/80	1/160	1/320	1/640	
Control PR 8	1/2	+	+	+	+	+	0	0		0	0	0	0	0	0	0	
	1/4	+	+	+	+	+	+	0		0	0	0	0	0	0	0	
	1/8	+	+	+	+	+	+	0		0	0	0	0	0	0	0	
	1/16	+	+	+	+	+	0	0		0	0	0	0	0	0	0	
	1/32	0	0	+	+	0	0	0		0	0	0	0	0	0	0	
	1/64	0	0	0	0	0	0	0		0	0	0	0	0	0	0	
Control Asian	1/2	0	0	0	0	0	0	0		0	0	0	0	0	0	0	
	1/4	+	0	0	0	0	0	0		0	0	0	0	0	0	0	
	1/8	+	+	0	0	0	0	0		0	0	0	0	0	0	0	
	1/16	+	+	0	0	0	0	0		0	0	0	0	0	0	0	
	1/32	0	0	0	0	0	0	0		0	0	0	0	0	0	0	
	1/64	0	0	0	0	0	0	0		0	0	0	0	0	0	0	
Enzyme-treated PR 8	1/2	+	+	+	+	0	0	0		+	+	+	+	0	0	0	
	1/4	+	+	+	0	0	0	0		+	+	+	+	+	0	0	
	1/8	+	+	0	0	0	0	0		+	+	+	+	0	0	0	
	1/16	+	+	0	0	0	0	0		+	+	0	0	0	0	0	
	1/32	+	+	0	0	0	0	0		0	0	0	0	0	0	0	
	1/64	0	0	0	0	0	0	0		0	0	0	0	0	0	0	
Enzyme-treated Asian	1/2	+	+	0	0	0	0	0		+	+	+	+	+	0	0	
	1/4	+	+	0	0	0	0	0		+	+	+	+	+	0	0	
	1/8	+	+	0	0	0	0	0		+	+	+	+	+	+	0	
	1/16	0	+	0	0	0	0	0		+	+	+	+	+	0	0	
	1/32	0	0	0	0	0	0	0		0	+	+	0	0	0	0	
	1/64	0	0	0	0	0	0	0		0	0	+	0	0	0	0	

Antiserum to enzyme-treated virus fixed complement in the presence of untreated virus; its titre in the presence of one unit of enzyme-treated virus antigen (*E* suspension diluted 1/8) was 1/160.

Antiserum to untreated PR8 influenza virus did not inhibit four haemagglutinating units of Asian influenza, neither did antiserum to enzyme-treated virus. However, it fixed complement in the presence of enzyme-treated and untreated Asian influenza virus suspensions, but its titre in the presence of either of these antigens did not exceed 1/20. Antiserum to enzyme-treated virus fixed complement in the presence of enzyme-treated Asian influenza virus suspension but not in the presence of untreated virus. The enzyme-treated virus antiserum titre in the presence of one unit of that antigen (*E* suspension diluted 1/8) was 1/320.

Neither control antiserum nor enzyme-treated virus antiserum fixed complement in the presence of standard soluble A antigen, or of caseinase C control solution.

Fig. 3. Neutralization of haemagglutination inhibiting activity of anti-PR 8 rabbit serum by various dilutions of PR 8 virus suspension incubated for 4 hr at 37°, μ 0.01, pH 7, with or without caseinase C and by fractions of these suspensions obtained after 1 hr centrifugation at 31,000 g in swinging bucket. *E* suspension: enzyme-treated suspension; *C* suspension: control suspension; *T* suspension: 2 ml. top supernatant; *M* suspension: 2 ml. medium supernatant; *B* suspension: pellet resuspended in 1.25 ml. bottom fluid. *B* fractions dilutions expressed as dilution of material sedimenting by 1 hr centrifugation at 31,000 g. *T* and *M* fractions dilutions expressed as dilution of material non-sedimented after 1 hr centrifugation at 31,000 g.

Evidence for virus breakdown

PR8 *E* suspension, 0.7 ml., was diluted 1/10 in saline. Five ml. of this dilution were centrifuged for 1 hr at 31,000 g in swinging bucket rotor SW39 of a Spinco model L centrifuge. After centrifugation, the 5 ml. volume was carefully divided into three fractions with the aid of a syringe and needle. Four ml. of the supernatant fluid were removed as two fractions, 2 ml. of top supernatant (*T*) and 2 ml. of medium

Table 6. Complement fixation after overnight incubation at 4° by various dilutions of concentrated purified PR8 suspensions treated or untreated by caseinase C and by fractions of these suspensions obtained after 1 hr centrifugation at 31,000 g in swinging bucket

PR 8 antigen	Antigen concentration	Complement fixation in the presence of guinea-pig serum													
		Control PR 8 antiserum diluted							Enzyme-treated PR 8 antiserum diluted						
		1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/10	1/20	1/40	1/80	1/160	1/320	1/640
C suspension	1/10	+	+	+	+	+	+	+	0	0	0	0	0	0	0
	1/20	+	+	+	+	+	+	+	0	0	0	0	0	0	0
	1/40	+	+	+	+	+	+	+	0	0	0	0	0	0	0
	1/80	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T from C	1/10	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1/20	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1/40	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1/80	0	0	0	0	0	0	0	0	0	0	0	0	0	0
M from C	1/10	+	+	+	+	+	+	+	0	0	0	0	0	0	0
	1/20	0	+	+	+	+	+	+	0	0	0	0	0	0	0
	1/40	0	0	+	+	+	+	+	0	0	0	0	0	0	0
	1/80	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B from C	1/2.5	+	+	+	+	+	+	+	0	0	0	0	0	0	0
	1/5.0	+	+	+	+	+	+	+	0	0	0	0	0	0	0
	1/10.0	+	+	+	+	+	+	+	0	0	0	0	0	0	0
	1/20.0	+	+	+	+	+	+	+	0	0	0	0	0	0	0
E suspension	1/40.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1/80.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1/10	+	+	+	+	0	0	0	+	+	+	+	0	0	0
	1/20	+	+	+	0	0	0	0	+	+	+	+	0	0	0
T from E	1/40	+	+	+	0	0	0	0	0	0	0	0	0	0	0
	1/80	0	+	+	0	0	0	0	0	0	0	0	0	0	0
	1/10	+	+	0	0	0	0	0	+	0	0	0	0	0	0
	1/20	+	+	0	0	0	0	0	+	0	0	0	0	0	0
M from E	1/40	+	+	0	0	0	0	0	0	0	0	0	0	0	0
	1/80	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1/10	+	+	0	0	0	0	0	+	0	0	0	0	0	0
	1/20	+	+	0	0	0	0	0	0	0	0	0	0	0	0
B from E	1/40	+	+	0	0	0	0	0	0	0	0	0	0	0	0
	1/80	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1/2.5	+	+	+	+	0	0	0	+	+	+	+	+	0	0
	1/5.0	+	+	+	+	0	0	0	+	+	+	+	+	0	0
	1/10.0	+	+	+	+	0	0	0	+	+	+	+	+	0	0
	1/20.0	0	0	0	0	0	0	0	+	+	+	+	+	0	0
	1/40.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1/80.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

E suspension: PR 8 suspension incubated for 4 hr at 37°, pH 7, μ 0.01 in the presence of caseinase C. C suspension: PR 8 control suspension. T: 2 ml. top supernatant; M: 2 ml. medium supernatant; B: pellet in 1.25 ml. bottom fluid. Concentration as dilution of uncentrifuged suspension.

supernatant (M). To the remaining bottom 1 ml., 0.25 ml. saline was added and the pellet resuspended in this 1.25 ml. volume.

In this fraction the concentration of material sedimentable by 1 hr of centrifugation at 31,000 *g* was equal to 1/2.5 the concentration of this material in the *E* suspension, whereas the concentration of material not sedimentable by 1 hr at 31,000 *g* was 1/12.5 the concentration of this material in *E* suspension. Three fractions of PR8 *C* suspension were obtained following the same procedure. The three fractions of both *E* and *C* suspensions were tested for ability to neutralize haemagglutination-inhibiting activity of anti-PR8 rabbit serum and for ability to fix complement in the presence of antisera to enzyme-treated and untreated virus. The activities of these fractions were compared with the activities of *E* and *C* suspensions, the concentration of each fraction being expressed as dilution of material present in corresponding uncentrifuged suspension.

T, M and B fractions of PR8 *E* suspension (when the concentration of non-sedimentable material present in B fraction is allowed for) neutralized haemagglutination-inhibiting activity of anti-PR8 rabbit serum as well as did *E* suspension itself. B fraction only of *C* suspension neutralized haemagglutination-inhibiting activity of this antiserum to the same extent as did *C* itself. Nothing but traces of neutralizing material were found in T and M fractions of *C* suspension (Fig. 3).

Complement-fixing activity in the presence of antiserum to untreated virus was found in all three fractions of *E* suspension. In the presence of one unit of T fraction of *E* antigen (T from *E* suspension diluted 1/40) or one unit of M fraction of *E* antigen (M from *E* suspension diluted 1/40) the titre of control antiserum was 1/20, whereas in the presence of one unit of B fraction of *E* antigen (B from *E* suspension diluted 1/20) its titre was 1/80. Complement-fixing activity in the presence of antiserum to enzyme-treated virus was found in B fraction only of *E* suspension. The titre of this serum in the presence of one unit of B fraction of *E* antigen (B from *E* suspension diluted 1/10) was 1/80.

The complement-fixing activity of the control (untreated) suspension was bound to material sedimenting at 31,000 *g*: no activity was bound in T fraction of *C* suspension, traces in M fraction and maximum activity in B fraction (Table 6).

DISCUSSION

Kuroya *et al.* (1957) and Skryabin (1957) reported that some actinomycetes produce in their culture filtrates substances displaying antibacterial activity and activity against influenza virus. Nevertheless, to our knowledge, none of these antiviral substances has been purified and used for studies similar to those described here. In our conditions the action of the antiviral preparation on infectivity and haemagglutinating activity cannot be attributed to a modification of sensitive cells. Therefore, the discussion will deal with a direct action on the virus particle *in vitro*.

The enzyme preparation used in these studies is proteolytic, as shown by digestion of casein, and may be a trypsin-like enzyme. Trypsin is known to act on influenza virus; indeed it has been reported (Mayrow, Robert, Winzler & Rafelson, 1961) that the neuraminidase and haemagglutinating activities of influenza A virus can be separated after prolonged treatment at pH 7 by a high concentration of crystalline trypsin. However, the enzymic activity of the Lee strain of influenza B virus, can

be decreased by crude or purified trypsin at pH 7.2 or pH 8.5. The same effect was observed with the Melbourne strain of influenza A virus, after trypsin treatment at pH 8.5 (Stone, 1949). The haemagglutinating activity of influenza virus is known to be fairly resistant to trypsin treatment, its diminution requiring either prolonged incubation at 37° and pH 7 (Gresser & Enders, 1961) or pH 8-9 (Cleeland & Sugg, 1963). This behaviour was confirmed by incubating the concentrated purified PR8 virus at 37° for 4 hr in a solution of crystalline trypsin (pH 7, μ 0.01, 18.5 μ g./ml. final volume) able to digest casein at the same rate as caseinase C at the usual concentration. After such a treatment, the haemagglutinating property of the virus was slightly increased. It seems therefore very unlikely that a trypsin-like proteolytic activity of the antiviral preparation is involved in the destruction of the haemagglutinating activity of the virus. The existence of a gradient in the sensitivity of the various viral properties towards the enzyme preparation under our conditions suggests either distinct enzymes responsible for their specific inactivation, or the progressive modification of the virus particle by a single enzyme.

The inability of treated virus to agglutinate red cells is associated with its inability to adsorb on red cells, which may account for the loss of infectivity of the virus particle. Production of haemagglutination inhibiting antibodies without antibodies fixing complement in the presence of virus particles, and appearance of a new antigenic activity together with evidence of breakdown of the virus raise several questions which are the subject of further investigation. Is the structure responsible for the production of antibodies which fix complement in the presence of viral particles destroyed? Is that structure, the one which is responsible for neutralizing haemagglutination-inhibiting antibodies and which is not sedimented at 31,000 g, the one which is also able to give haemagglutination-inhibiting antibodies and to react with strain specific complement-fixing antibodies that it cannot produce? Is the new antigenic activity detected in treated virus suspension, in that material which is sedimented at 31,000 g, the consequence of the unmasking of a pre-existing antigen or of a chemical change induced by the enzyme treatment? This antigen cannot be the soluble antigen since antiserum to enzyme-treated virus does not react with standard soluble influenza A virus antigen. Nevertheless, data support the hypothesis of the unmasking of an antigen present in both PR8 and Asian influenza virus particles. V antigens are known to possess some components cross-reacting within types in addition to strain specific ones (Henle & Wiener, 1944; Fulton & Dumbell, 1949). Sera obtained from animals or man after consecutive influenza infections show recall between distantly related strains (Henle, Lief & Fabiyi, 1958; Hobson & Pearson, 1961). Non-specific recalls are troublesome when preparing pure anti-S serum (Lief, 1963). Our control PR8 guinea-pig antiserum diluted 1/20 fixed complement in the presence of purified Asian influenza virus, but its titre in the presence of purified PR8 was much higher.

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