Release of Influenza Virus Neuraminidase by Caseinase C of *Streptomyces albus* G

By MONIQUE REGINSTER

*Laboratoire de Microbiologie générale et médicale
Université de Liège, Liège, Belgium*

(Received 30 June 1965)

SUMMARY

When concentrated purified influenza virus PR 8 was incubated at 37° with caseinase C in phosphate buffer at pH 7 and ionic strength 0.01 or 0.1, the neuraminidase activity was released from the virus particle and progressively destroyed. Under these conditions a material was released from virus which neutralized the haemagglutination inhibiting antibodies of anti-PR 8 rabbit serum. This material and neuraminidase, released by caseinase C, were both sedimented by centrifugation at 125,000 *g*. Neither was adsorbed to fowl red cells. After 4-hr incubation with caseinase C in phosphate buffer at pH 7 and ionic strength 0.1, the infectivity of influenza virus PR 8 suspension was considerably decreased, but its haemagglutinating activity was unchanged and remained bound to the virus particles. Under these conditions, most of the surface projections of the virus particles were removed.

INTRODUCTION

We reported (Reginster, 1965*a*) that when influenza virus PR 8 was incubated for 4 hr at 37° with caseinase C in ionic strength 0.01 pH 7 phosphate buffer, the infectivity, the haemagglutinating activity and the neuraminidase activity were destroyed and the serological properties of the virus suspension were modified. When the time of incubation was decreased to 1 hr, the infectivity and the haemagglutinating activity decreased significantly, but the neuraminidase was unchanged (Reginster, 1965*a*). Caseinase C alters the surface of virus particle, removing its typical projections (Reginster, 1965*b*) and liberating at least two fragments distinguishable by their sedimentation constants and biological properties (Reginster, 1965*c*). In the present paper, we describe the effect of caseinase C on influenza virus PR 8 neuraminidase and discuss the relationship between neuraminidase, haemagglutinating activity, serological properties and infectivity of virus PR 8.

METHODS

Concentration and purification of virus PR 8, production of anti-PR 8 rabbit serum and techniques for measuring haemagglutinating activity, infectivity, neuraminidase activity and ability to neutralize haemagglutination inhibiting antibodies were as described by Reginster (1965*a*). Two preparations of caseinase C, kindly supplied by Dr J. M. Ghuyssen, were used; they behaved identically. Incubations of virus PR 8 with caseinase C were done in pH 7 phosphate buffer, at either 0.01 or 0.1 ionic strength (μ). Caseinase C was used at a final concentration

of 35 caseinolytic units. One caseinolytic unit was defined as the amount of enzymic preparations which digests 50 % of the casein present in 0.5 ml. of standard solution (0.2 % soluble casein in 0.05 M- K_2PO_4) within 15 min. of incubation at 37°. The casein left after enzymic digestion was measured by trichloroacetic acid precipitation (4.5 ml. of 1 % trichloroacetic acid solution added to the 0.5 ml. assay). Untreated control suspensions of virus PR 8 were obtained by incubating the virus in buffer without caseinase C. The neuraminidase activity of material obtained after incubation of virus PR 8 with caseinase C or after high speed centrifugation of untreated suspensions was expressed as the concentration of control suspension which would have the same neuraminidase activity. This concentration, expressed as a percentage of the undiluted control suspension, was graphically determined in each experiment from the curve showing the neuraminidase activity of the corresponding control suspension as a function of virus concentration (Reginster, 1965*a*).

Pellets and supernatant fluids from virus suspensions were obtained by centrifugation for 1 hr at 81,000*g* in the swinging bucket rotor SW 89 of a Spinco model L centrifuge (20,000 rev./min.) The 5 ml. tubes were filled with undiluted suspensions adjusted, when necessary to μ 0.1 by adding concentrated phosphate buffer (pH 7) or with virus suspensions diluted 1/5 in saline. The supernatant fluids were collected with a syringe and needle. The activities of the supernatant fluids were measured in the 4 ml. first collected, the bottom 1 ml. being discarded. The pellets were resuspended in a volume of diluent suitable for comparison with the corresponding supernatant and with the uncentrifuged suspension of virus.

Adsorption to red cells was done as follows. Samples of virus suspensions, untreated by caseinase C, or samples of supernatant fluids and pellets from these suspensions, were diluted 1/5 in saline and mixed with 20 % (v/v) packed fowl red cells. After 15 min. at room temperature, mixtures were centrifuged (15 min. at 1500*g*) and the supernatant fluids collected.

RESULTS

Release of neuraminidase

Concentrated purified virus PR 8 was incubated at 37° for various times with caseinase C at μ 0.01 or μ 0.1; corresponding control suspensions were prepared. The neuraminidase activity was measured, either in the uncentrifuged caseinase C treated virus suspensions, in the supernatant fluids or in the pellets of caseinase C treated and control untreated suspensions (Table 1). When collecting supernatant fluids and pellets of control virus suspensions, about 60 % of the neuraminidase activity of the uncentrifuged suspensions was recovered; about 90 % of this activity was associated with the pellet.

The neuraminidase activity of virus suspensions previously incubated for 1 hr or less at μ 0.01 or μ 0.1 with caseinase C was equal to, or higher than, the neuraminidase activity of untreated suspensions. When these suspensions were centrifuged, all the neuraminidase activity was recovered and was associated up to 90 %, with the supernatant fluid. After incubation for 2 hr or more with caseinase C at μ 0.01, the neuraminidase activity decreased significantly. When the incubation with caseinase C lasted 90 min. or more at μ 0.1, the neuraminidase activity (whole

Table 1. Neuraminidase activity of PR 8 virus suspension incubated at 37° in phosphate buffer (pH 7; μ 0.01 or μ 0.1) with or without caseinase C; and of supernatant fluids and pellets of these suspensions, unadsorbed or adsorbed by fowl red cells

Expt. (no.)	Time of incubation (min.)	Ionic strength (μ)	Neuraminidase activity* after incubation									
			Without caseinase C					With caseinase C				
			Supernatant		Pellet			Supernatant		Pellet		
			U†	A‡	U†	A‡	Suspension	U†	A‡	U†	A‡	A‡
6	30	0.01	.	.	.	.	108	122	.	15	.	.
2	60	0.01	.	.	.	.	150	.	.	.	.	.
3	60	0.01	.	.	.	.	155	.	.	.	.	.
4	60	0.01	.	.	.	.	110	100	.	< 1	.	.
1	60	0.01	.	.	56	.	90	.	.	3	.	.
5	60	0.01	.	.	.	.	140	145	.	1	< 1	.
2	120	0.01	.	.	.	.	46	.	.	.	.	.
3	120	0.01	.	.	.	.	52	.	.	.	.	.
5	120	0.01	.	.	.	.	70	.	.	.	.	.
2	240	0.01	.	.	.	.	3	.	.	.	.	.
3	240	0.01	.	.	.	.	36	.	.	.	.	.
4	240	0.01	.	.	.	.	36	.	.	.	.	.
5	240	0.01	.	.	.	.	23	.	.	.	.	.
7	240	0.01	4	.	64	.	.	.	.	.	.	.
4	360	0.01	.	.	.	.	12	.	.	.	.	.
8	60	0.1	.	.	.	.	.	140	140	19	1	.
12	60	0.1	.	.	.	.	.	62	55	30	9	.
6	90	0.1	13	< 1	.	.	119	76	.	32	< 1	.
8	120	0.1	.	.	.	.	.	100	145	7	< 1	.
8	240	0.1	1	< 1	50	1	.	100	84	4	< 1	.
9	240	0.1	4	< 1	60	10	.	.	.	.	.	.
11	240	0.1	.	.	.	.	.	25	.	< 1	.	.

* As concentration (%) of virus suspension pre-incubated without caseinase C which would have the same neuraminidase activity; † Unadsorbed by fowl red cells; ‡ Adsorbed by fowl red cells.

Table 2. *Haemagglutinating activity of PR 8 virus suspension incubated at 37° in phosphate buffer (pH 7; μ 0.01 or μ 0.1) with or without caseinase C; and of supernatant fluids and pellets of these suspensions, unadsorbed or adsorbed by fowl red cells*

Expt. (no.)	Time of incubation (min.)	Ionic strength (μ)	Haemagglutinating activity* after incubation										
			Without caseinase C				With caseinase C						
			Suspension A†	Supernatant		Pellet		Suspension U‡	Supernatant		Pellet		
			U‡	A†	U‡	A†	U‡	A†	U‡	A†	U‡	A†	
6	30	0.01	.	.	.	.	.	100	8	.	.	100	.
6	90	0.01	< 1	.	.	.	.	.	.	.	.	.	.
7	240	0.01	.	4	.	100	.	.	.	.	.	.	.
8	60	0.1	.	.	.	.	.	.	4	< 1	.	100	< 1
12	60	0.1	.	.	.	.	.	100	3	< 1	.	100	< 1
6	90	0.1	< 1	.	.	.	.	100	11	.	.	.	.
8	120	0.1	.	—	.	.	.	.	4	< 1	.	100	< 1
10	240	0.1	.	.	.	.	.	100	.	.	.	.	.
8	240	0.1	.	.	.	.	.	.	6	< 1	.	100	< 1
9	240	0.1	.	2	< 1	100	.	.	.	.	.	100	.
7	240	0.1	.	4	.	100	.	.	9	.	.	100	.
11	240	0.1	.	.	.	.	.	.	.	.	.	.	.

* As % of the haemagglutinating activity of the virus suspension incubated for the same time in the same buffer without caseinase C; † adsorbed by fowl red cells; ‡ unadsorbed by fowl red cells.

virus suspension, or supernatant plus pellet) was unchanged in 5 out of 6 experiments. The neuraminidase activity bound to the virus particles diminished as the length of incubation with caseinase C increased (Table 1, Expt. 8). After 60 min. with caseinase C at μ 0.01, there was very little neuraminidase bound to the virus particles (Table 1, Expts. 1, 4, 5). Nevertheless, after 60 or 90 min. with caseinase C at μ 0.1, the neuraminidase activity associated with the virus particles corresponded to the activity of the untreated control suspension diluted from about 1/5 to 1/3 (Table 1, Expts. 6, 8, 12).

Ability of neuraminidase to adsorb to red cells

Concentrated purified virus PR 8 was incubated at 37° with or without caseinase C at μ 0.1. These suspensions were centrifuged and the neuraminidase activity of supernatant fluid or pellet, or of both, was measured before and after red cells adsorption. The neuraminidase activity in the pellets of untreated control suspensions was decreased about 10-fold by red-cell adsorption and the low activity found in the supernatant fluid of the same suspensions was completely removed (Table 1, Expt. 8, 9). Red-cell adsorption did not modify significantly the neuraminidase activity of the supernatant fluids of virus suspensions pre-incubated with caseinase C, but it abolished or decreased considerably the neuraminidase activity found in the pellets of the corresponding control suspensions (Table 1, Expts. 6, 8, 12).

Table 3. *Infectivity of PR 8 virus incubated at 37° for 4 hr in phosphate buffer (pH 7; μ 0.1) with or without caseinase C*

Expt. (no.)	Infectivity after incubation			
	Without caseinase C		With caseinase C	
	log. ID 50 in 1 ml.	Standard error	log. ID 50 in 1 ml.	Standard error
10	6.15	± 0.11	4.55	± 0.15
11	8.48	± 0.11	5.06	± 0.13

Properties of PR 8 virus suspensions after release of neuraminidase

Haemagglutinating activity. Concentrated purified virus PR 8 was incubated at 37° with or without caseinase C, at μ 0.01 and at μ 0.1. The haemagglutinating activity was measured in these suspensions, in their supernatant fluids and pellets, adsorbed and unadsorbed by fowl red cells (Table 2). After 30 min. at μ 0.01, and after 1, 2 or 4 hr at μ 0.1, the haemagglutinating activity of the virus suspensions incubated with caseinase C was the same as that of the control suspensions. The haemagglutinating activity of all these suspensions was bound to material in the pellets. The supernatant fluids contained less than 10% of the haemagglutinating activity of the corresponding uncentrifuged suspensions. Red-cell adsorption decreased considerably the haemagglutinating activity of any virus suspension, supernatant fluid or pellet material as well.

Infectivity. Concentrated purified virus PR 8 was incubated for 4 hr at 37° with or without caseinase C at μ 0.1 (Table 3). The infectivity of the virus incubated with caseinase C was < 5% the infectivity of the virus incubated in buffer alone.

Release of material neutralizing haemagglutination inhibition. Concentrated purified virus PR 8 was incubated at 37° in μ 0.1 buffer for 1, 2 or 4 hr with caseinase C, and for 4 hr without caseinase C. Haemagglutination inhibition neutralizing, haemagglutinating and neuraminidase activities were titrated in the supernatant fluids of these suspensions (Table 1, 2 Expt. 8). There was twice as much haemagglutination inhibition neutralizing material in the supernatant fluid of the virus suspension pre-incubated for 4 hr with caseinase C as in the supernatant of the same suspension incubated for only 1 hr with caseinase C, and 4 times as much as in the supernatant

Fig. 1. Neutralization of haemagglutination inhibiting antibodies of PR 8 rabbit anti-serum by the supernatant fluid from virus PR 8 suspension pre-incubated at 37° for 4 hr. in μ 0.1, pH 7 phosphate buffer (I); or for 1 hr. (II), 2 hr. (III), 4 hr. (IV) in μ 0.1, pH 7 phosphate buffer+caseinase C (see Expt. no. 8). ▨, Haemagglutination in the presence of diluent; □, in the presence of supernatant fluid adsorbed; ■, in the presence of supernatant fluid unadsorbed.

fluid of the untreated suspension (Fig. 1). Red-cell adsorption abolished the activity of the supernatant fluid of the control suspension, decreased by 50% the activity of the supernatant fluid of the virus suspension pre-incubated for 1 hr with caseinase C, but did not decrease the activity of the supernatant fluid of virus suspensions treated for 2 or 4 hr with caseinase C (Fig. 1).

Sedimentation of neuraminidase and haemagglutination inhibition neutralizing activities from supernatant of caseinase C treated virus suspension

The sedimentation was done twice, by centrifugation at 125,000g (39,000 rev./min., swinging bucket rotor S.W. 39, Spinco Model L centrifuge) of 5 ml. supernatant fluid from concentrated purified virus PR 8 pre-incubated for 1 hr with caseinase C. In one experiment (Tables 1, 2, Expt. 5), the supernatant fluid was obtained after caseinase C treatment at μ 0.01; it was centrifuged for 10 hr at 125,000g. In the other experiment (Tables 1, 2, Expt. 12), the supernatant fluid was obtained after caseinase C treatment at 0.1; it was centrifuged for 12 hr at 125,000g. In both cases, a thin pellet was obtained. The supernatant fluids were collected with a syringe and needle. In experiment number 5, 4 ml. of supernatant were removed and the pellet was resuspended in the 1 ml. fluid left in the tube. In experiment number 12, 4.5 ml. of supernatant were removed and the pellet resuspended in 0.5 ml. bottom fluid. The haemagglutination inhibition neutralizing and neuraminidase activities of these fractions were measured and compared to the activities of samples of the corresponding preparations kept at 4° during the prolonged high-speed centrifugation (Table 4). In experiment number 5, 100 % of the neuraminidase and 80 % of the haemagglutination inhibition neutralizing activities were recovered; bottom fractions contained 100 % of the neuraminidase activity and 72 % of the haemagglutination inhibition neutralizing activity. In experiment number 12, 100 % of the neuraminidase and 90 % of the haemagglutination inhibition neutralizing activities were recovered; bottom fraction contained 80 % of the neuraminidase activity and 75 % of the haemagglutination inhibition neutralizing activity.

Table 4. *Sedimentation of neuraminidase and haemagglutination inhibition neutralizing activities released by caseinase C treatment of PR 8 virus*

Fraction collected after 125,00g centrifugation	Neuraminidase activity*		Haemagglutination inhibition neutralizing activity*	
	Expt. 5†	Expt. 12‡	Expt. 5†	Expt. 12‡
Supernatant fluid	2	10	35	15
Bottom fraction	102	72	80	75

* As % of the activity of the material not centrifuged at 125,000g; † 10-hr centrifugation at 125,000g; ‡ 12-hr centrifugation at 125,000g.

Morphology of the virus particles treated by caseinase C at μ 0.1

Concentrated purified PR 8 virus was incubated for 1 or 4 hr at 37° with caseinase C at μ 0.1. After this treatment, the virus particles were sedimented by centrifugation for 1 hr at 31,000g. They were then resuspended in water, mixed with an equal volume of potassium phosphotungstate solution (2%, pH 7) and examined with a Siemens Elmiskop I electron microscope. Controls for infectivity, neuraminidase activity and haemagglutinating activity were done in parallel in the 4 hr experiment (Tables 1-3, Expt. 11). Haemagglutinating activity and neuraminidase activity only were checked in the 1 hr incubation experiment (Tables 1 and 2, Expt. 12) but the neuraminidase and the haemagglutination inhibition neutralizing

material released by the virus particles were sedimented by 12 hr centrifugation at 125,000g (Table 4).

After 4 hr incubation, some virus particles looked unaltered, some had lost almost all their surface projections. The majority had fewer projections than normal virus particles (Pl. 1A). After 1 hr incubation, most virus particles had no obvious alteration of their surface, although some had lost a few projections (Pl. 1B).

DISCUSSION

By incubating influenza virus PR 8 with caseinase C at two ionic strengths (Reginster 1965*a*, and this paper) we found quantitative but not qualitative differences in the modifications of the virus properties. This does not imply that all the effects of the caseinase C preparations are due to one enzyme. Haemagglutination is obviously related to the surface projections of the virus. Indeed, when electron micrographs showed virus particles completely lacking their normal surface projections (Reginster, 1965*b*) the haemagglutinating activity was considerably decreased. Nevertheless, the surface of most of the virus particles can be considerably freed from surface projections without modification of haemagglutinating ability as measured by the standard technique. This fits in well with the idea that the surface of the virus is made up of repeating units, two of which, when suitably located, would suffice to link two red cells together.

By high speed centrifugation we succeeded in concentrating at the same rate free neuraminidase and haemagglutination inhibition neutralizing material. Thus caseinase C split up superficial layers of influenza virus either into subunits differing in their biological properties but sedimenting under the same conditions, or more likely into subunits carrying at least neuraminidase and haemagglutination inhibition neutralizing activities. Sedimentation analysis revealed the presence of 6 S material in virus PR 8 suspension treated for 1 hr by caseinase C at μ 0.01 (Reginster, 1965*c*). The weight of one surface projection, calculated on the basis of Horne & Wildy's mensurations (1963) and by assuming that it is made of protein, is 90,000. It appears from Greenberg's table (1951) that this weight is not inconsistent with a sedimentation constant of 6 S. Haemagglutination, although related to the surface projections, does not seem to be located on the site responsible for neutralization of haemagglutination inhibition or for neuraminidase activity since adsorption to red cells did not modify these activities when they were released from the virus particles by caseinase C. This would mean that the inhibition of haemagglutination by antibodies is a 'steric' one (Fazekas de St Groth, 1963) since the antigenic sites responsible for fixation of antibodies do not adsorb to red cells. The question of identity or non-identity of the sites responsible for neuraminidase activity and neutralization of haemagglutination inhibition still remains unanswered. It has been, so far, impossible to determine what degree of integrity of the virus surface is necessary for full infectivity of the virus particle because accurate quantitative data are lacking.

Mayron, Robert, Winzler & Rafelson (1961) obtained separation of neuraminidase from virus PR 8 by trypsin treatment. This neuraminidase did not haemagglutinate nor block agglutination of red cells by 'indicator' Lee virus. It has been, so far, impossible to establish complete comparison between the effects of trypsin and

a

b

of caseinase C. Nevertheless, trypsin is able to release surface projections of an influenza virus strain highly sensitive to its action (Borecký, Lackovič & Mrena, 1964), as is caseinase C action on PR 8 strain.

We thank Dr C. M. Calberg-Bacq for electron microscope examination, Mr L. Dierickx for determining the caseinolytic activity of enzymic preparations, Mr J. L. Renson and Miss D. Marique for technical assistance. We are indebted to the Fonds de la Recherche Scientifique Médicale for a grant in aid.

REFERENCES

- BORECKÝ, L., LACKOVIČ, V. & MRENA, E. (1964). The effect of trypsin on influenza virus. *Acta virol.* **8**, 555.
- FAZEKAS DE ST GROTH, S. (1963). Steric inhibition: neutralization of a virusborne enzyme. *Ann. N.Y. Acad. Sci.* **103**, 674.
- GREENBERG, D. M. (1951). *Amino-acids and Proteins*. Springfield: Thomas.
- HORNE, R. W. & WILDY, P. (1963). *Advances in Virus Research*. New York: Academic Press.
- MAYRON, L. W., ROBERT, B., WINZLER, R. T. & RAFELSON, M. E. JUN. (1961). Studies on the neuraminidase of influenza virus. I. Separation and some properties of the enzyme from Asian and PR 8 strains. *Arch. Biochem. Biophys.* **92**, 475.
- REGINSTER, M. (1965*a*). Inactivation of influenza virus by caseinase C from *Streptomyces albus* G culture-filtrate. *J. gen. Microbiol.* **40**, 157.
- REGINSTER, M. (1965*b*). Modification of influenza virus morphology by *Streptomyces albus* G caseinase C. *Acta virol., Prague*, **9**, 462.
- REGINSTER, M. (1965*c*). Sedimentation analysis of influenza virus suspension treated by caseinase C from *Streptomyces albus* G. *Life Sci.* **4**, 445.

EXPLANATION OF PLATE

Influenza virus PR 8 after incubation at 37° in pH 7, μ 0.1 phosphate buffer with caseinase C. Magnification: $4,4 \times 40,000$.

A. 4-hr incubation. B. 1-hr incubation.