BIKEN'S JOURNAL 1, 151-163 (1959)

Gamma Ray Inactivation Analysis of Infective Particles in von Magnus type Incomplete Preparation of Influenza Virus

TETSUO YOSHISHITA

Department of Preventive Medicine, Research Institute for Microbial Diseases Osaka University, Osaka

(Received for publication, September 30, 1959)

SUMMARY

Infective agents in a von Magnus type incomplete virus preparation resulting from serial undiluted passage through PR8 infected chorioallantoic fluid was inactivated by gamma ray irradiation showing a multiple hit curve. This furnishes strong contrast to the fact that the inactivation of standard passage virus follows a single hit curve. This difference in inactivation suggests that the infective agents contained in the third undiluted passage virus preparation have quite different properties and structures from those in standard passage preparations concerning with the development of the infectivity. This means the existence of a new criterion of the virus particles.

Supposing the existence of several infective centers in individual infective particles in undiluted passage virus this result can be rationally explained.

INTRODUCTION

Since von Magnus phenomenon (von Magnus, 1951) was first reported, various attempts have been presented to solve the problems of the mechanism of this phenomenon.

While there are marked differences of the properties of an incomplete virus preparation and a standard passage one, for example in their sedimentation constants, morphological appearance and nucleic acid contents, no one knows whether the quality of infective agents contained in incomplete virus preparations is same as that of standard fully active virus and no attempt has been made to analyse this point of the von Magnus phenomenon.

The author is convinced that radiation studies are the only fruitful way to analyse the structures and qualities of infective agents in an incomplete virus preparation.

This paper describes data supporting the existence of multiple infective centers in infective particles in an undiluted passage virus preparation.

An interpretation of the results is based upon the target theory throughout this work.

MATERIAL AND METHODS

Virus strain: PR8 strain of influenza virus was employed in this work. Freshly

harvested chorioallantoic fluid infected with egg-adapted virus was used as the starting inoculum.

Standard passage preparation and undiluted passage preparation: In case of standard passage, 0.2 ml. of infected allantoic fluid diluted to 10^{-7} with broth was inoculated into the chorioallantoic cavity of 11 day-old embryonated eggs and harvested after 48 hours incubation at 35.5°C. and 5 hours chilling at 4°C. To produce undiluted passage preparation 1 ml. of undiluted infected chorioallantoic fluid was applied to each egg and the virus was harvested in the same manner.

Infectivity titration: For the infectivity titration a 10 fold serial dilution was prepared with broth containing 1000 U./ml. of penicillin and 500 micrograms/ml. of streptomycin. Aliquots of 0.2 ml. of each dilution were inoculated into the chorioallantoic cavity of five to seven 11 day-old embryonated eggs. After 48 hours incubation at 35.5°C. the chorioallantoic fluid was tested for virus growth by the conventional hemagglutination test on a slide glass. The 50 per cent infective titer (EID_{50}) was calculated by the method of Reed and Muench and was expressed as the EID_{50} per 0.5 ml.

Hemagglutination titration: Salk's pattern method was used. Two series of serial two fold dilutions of the preparations from 2– and 3– fold dilution were made in 0.5 ml. of 1 per cent citrate saline. Then 0.5 ml. of 0.5 per cent suspension of thrice washed chicken red cells was added. The test mixtures were kept standing at room temperature for 2 hours.

The last tube giving definite agglutination was considered as the end point and the titer was expressed as the reciprocal of that dilution.

I/A ratio: The I/A ratio was calculated by dividing EID_{50}/ml. by the corresponding hemagglutinating titer/ml. of the sample and expressed as the logarithm of that quotient.

Fractionation by the sucrose density gradient column: A modification of Schaffer's method for the fractionation of poliovirus was used. Sucrose was dissolved in 0.14 M NaCl to give a series of solutions of varying specific gravity ranging from 1.039 to 1.133 (the corresponding percentages by weight of the solutions were 10, 18, 23, 27, and 31 per cent). Aliquots of 0.7 ml. of each solution were layered carefully with a J-tipped pipette into a 5 ml. Spinco lusteroid tube (12.5 × 65 mm.) from the bottom to the top starting with the heaviest solution. After standing over night at 0°—5°C. to obtain a continuous gradient, 0.5 ml. of virus sample was placed on the top of the sucrose density gradient column. Then the completed columns were centrifuged in a Spinco Model E ultracentrifuge with a SW-39 swinging bucket type rotor at 12,590 rpm. for 90 minutes. At the end of the run the liquid column was divided in eight fractions from the top to the bottom of the tube (Fraction 1, 2, ..., 8) using samplers.

Gamma ray irradiation: Virus preparations were ten fold diluted with broth and 1 ml. aliquots were distributed into 1 cm. × 1 cm. × 1.5 cm. cylindrical glass cups. These cups were placed in a Co^{60} irradiation apparatus (Type: Toshiba MI-102). The distance between the center of the Co^{60} source and the middle point of the sample liquid layer was 30 mm. For the adjustment of the irradiation distance a specially designed remote control attachment was used. The measurements showed that the intensity of the gamma rays from this Co^{60} source was 2176 röntgens per minutes at the 5 cm. distance from the center of the source. The gamma ray dosis for the inactivation was calculated from this value. At the irradiation position the samples received 5×10^5 röntgens in 80 minutes. After a desired period of irradiation, the samples were immediately assayed for their EID_{50}.

EXPERIMENTALS

Survival curve of standard passage virus:

Infected chorioallantoic fluid was centrifuged at 3000 rpm. for 10 minutes and 1 ml. of the supernatant was diluted with 9 ml. of neutral broth to eliminate the so-called secondary effects of ionizing radiation. Aliquots of the diluted chorioallantoic fluid were employed for the inactivation experiments. The experiments

were repeated over a period of several months. The survival curve of PR8 standard passage virus is presented in Fig. 1.

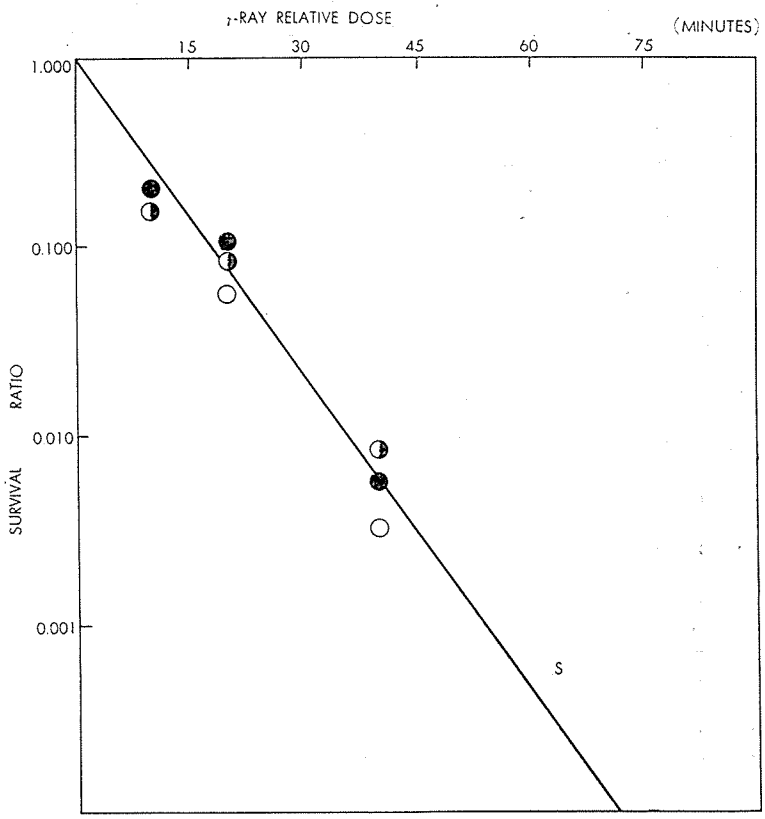

Fig. 1. Survival curve of the infective agents in standard passage chorioallantoic fluid of PR8

The results of several independent experiments are summarized in the figure and the solid line shows the theoretical survival ratio calculated from observed data. The survival ratio is plotted against gamma ray relative dosis.

The results show good agreement with the equation of single hit inactivation curve $N/N_0 = e^{-\alpha D}$, which means that one ionization at any point in the so-called infective center destroys the infectivity of the virus, where N/N_0 is the surviving fraction after a dose D is given and α is the characteristic constant for PR8 virus in the case of gamma ray inactivation.

Calculation of the 37 per cent survival doses indicated a diameter of about 35 millimicrons (as a spherical body) as the size of the infective center. Apparently this size does not coincide with the size of whole virus particles measured by electron microscopy, and this discrepancy suggests the infective core occupies only a part of the virus particles and the other parts are not responsible for infectivity

of the particles.

Survival curves of the infective agents in the second and third undiluted passage preparations;

The EID_{50} titration shows that the titers of second and third undiluted passage preparations are considerably high, and when the I/A ratio is calculated for these preparations the values are 3 or 4 (in logarithm unit) while that of standard preparations is about 6 showing that there are concomitant so-called non-infectious hemagglutinin in these preparations. This suggests that these preparations still contain many infective particles.

A series of experiments was carried out to inactivate the infective agents in second and third undiluted passage virus preparations applying the same techniques as in the case of standard virus preparation.

As presented in Fig. 2, these survival curve is a multiple hit type rather than a single hit type.

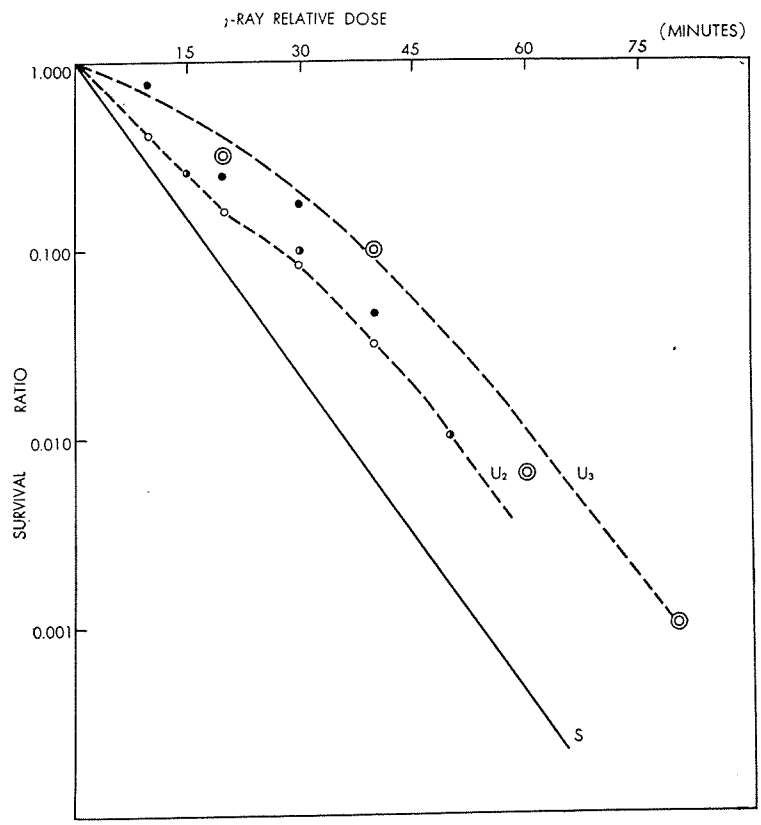

Fig. 2. Survival curves of infective agents in second and third undiluted chorioallantoic fluid. Solid line: Control (Standard passage preparation)

○ ◑ **Second undiluted possage preparation**

● ◎ **Third undiluted passage preparation**

These curves, however, do not completely fit any theoretical multiple hit curve. The survival curve of the second undiluted passage preparation showed a type transitional from the standard virus curve to the curves of third undiluted passage preparations.

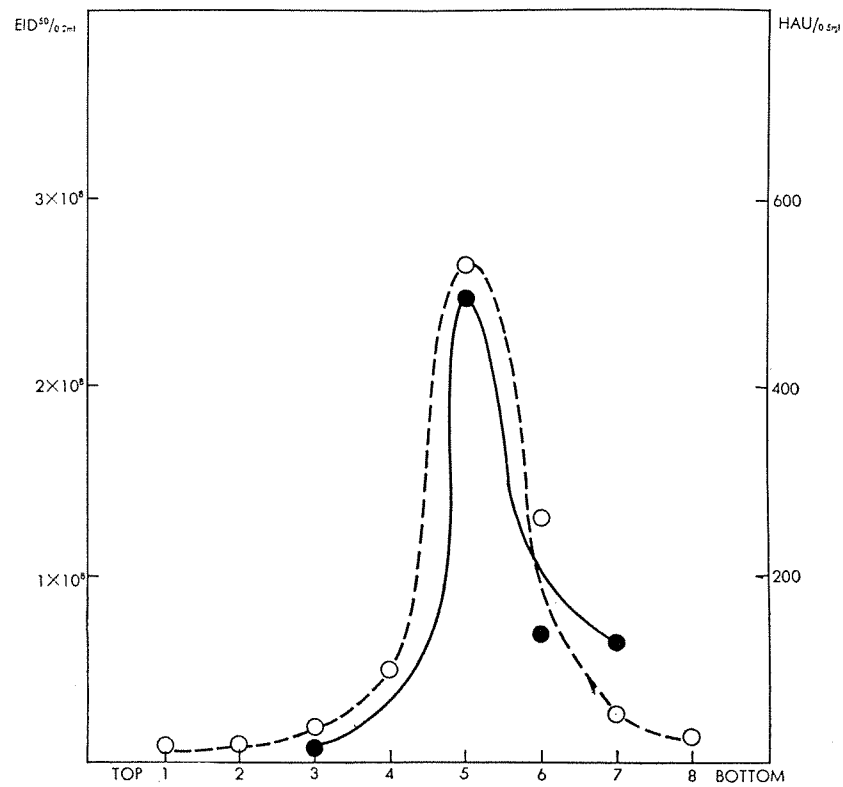

Fig. 3. Distribution of hemagglutinating and infective activity of a standard passage preparation in a sucrose density gradient column.
○ Hemagglutinating activity
● Infective activity

Table 1. EID_{50} and hemagglutinating titer of fractions obtained from a standard passage preparation

Fraction No.	EID_{50}/0.5ml.	Hemagglutination U./0.5ml.	I/A ratio
1	—	0	—
2	—	8	—
3	$10^{6.90}$	16	5.70
4	—	96	—
5	$10^{8.80}$	512	6.09
6	$10^{8.25}$	256	5.84
7	$10^{8.22}$	48	6.54
8	—	24	—

Fractionation of infective agents in the incomplete preparation:

A von Magnus type incomplete virus preparation could not be considered as homogeneous from the view point of morphological observations and sedimentation

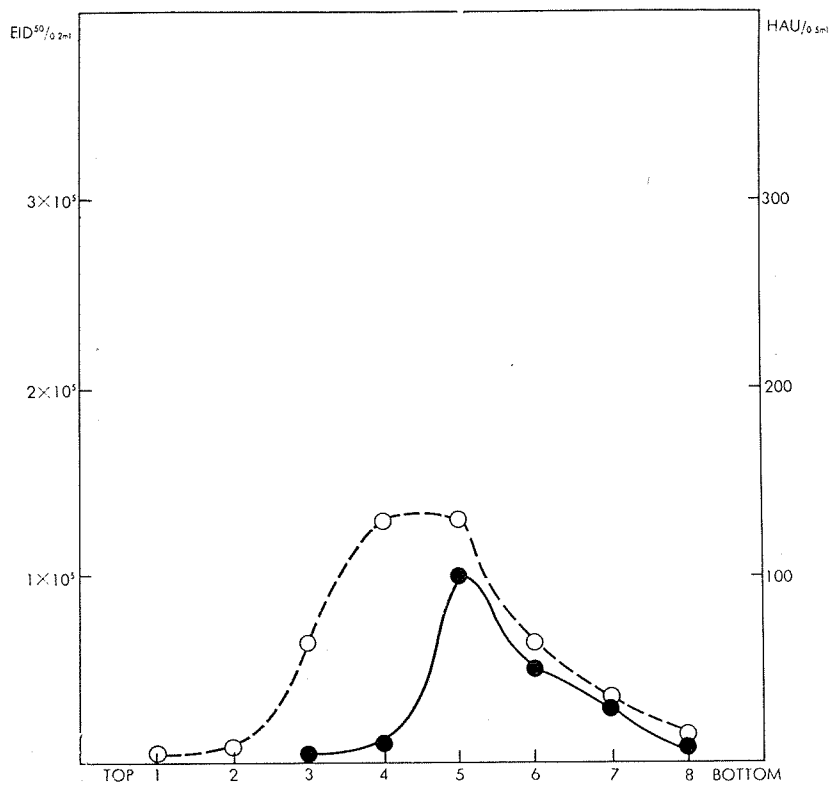

Fig. 4. Distribution of hemagglutinating and infective activity of a second undiluted passage preparation in a sucrose density gradient column.
○ Hemagglutinating activity
● Infective activity

Table 2. EID_{50} and hemagglutinating titer of the fractions obtained from a second undiluted passage preparation

Fraction No.	EID_{50}/0.5ml.	Hemagglutination U./0.5ml.	I/A ratio
1	$10^{1.92}$	8	1.02
2	$10^{2.65}$	8	1.75
3	$10^{3.55}$	64	1.74
4	$10^{4.40}$	128	2.29
5	$10^{5.40}$	128	3.29
6	$10^{5.07}$	64	3.26
7	$10^{4.90}$	32	3.40
8	$10^{4.12}$	16	2.92

experiments carried out in the past. Therefore, an incomplete preparation was fractionated by the density gradient method. Results on the distribution of hemag-

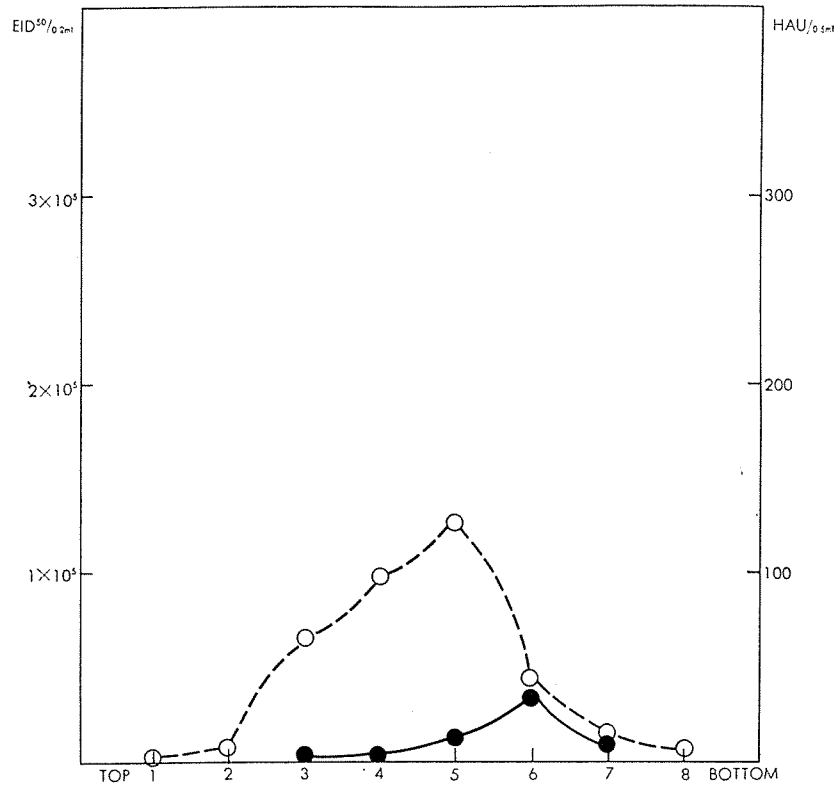

Fig. 5. Distribution of hemagglutinating and infective activity of a third undiluted passage preparation in a sucrose density gradient column.
○ Hemagglutinating activity
● Infective activity

Table 3. EID_{50} and hemagglutinating titer of fractions obtained from a third undiluted passage preparation

Fraction No.	EID_{50}/0.5ml.	Hemagglutination U./0.5ml.	I/A ratio
1	$10^{2.02}$	3	1.55
2	$10^{1.90}$	8	1.00
3	$10^{2.02}$	64	0.21
4	$<10^{2.90}$	96	<0.92
5	$10^{3.90}$	128	1.79
6	$10^{4.65}$	32	3.55
7	$10^{4.00}$	16	2.86
8		8	

glutinating activities and EID_{50} after centrifugation at 12,590 rpm. for 90 minutes are presented in Fig. 3, 4, and 5, and Table 1, 2, and 3.

In the case of standard passage preparations the sharp peaks of hemagglutinin and EID_{50} titer were both in the 5th fraction. This means roughly that the standard particles have hemagglutinating and infective activities simultaneously and the constitution of the population is very homogeneous. In the case of third undiluted passage preparation the dissociation between hemagglutinating and infective activity distribution is remarkable. Furthermore it is a very surprising fact that the peak of the infectivity titer is not present at the height of the fifth fraction, but the infectivity dominates in the sixth fraction.

Inactivation experiments of isolated fractions:

i) Survival curve of fifth and sixth fractions obtained from the third undiluted passage preparation:

As mentioned above, the survival curves of the infective agents in second and third undiluted passage preparations did not fit theoretical multiple hit curves.

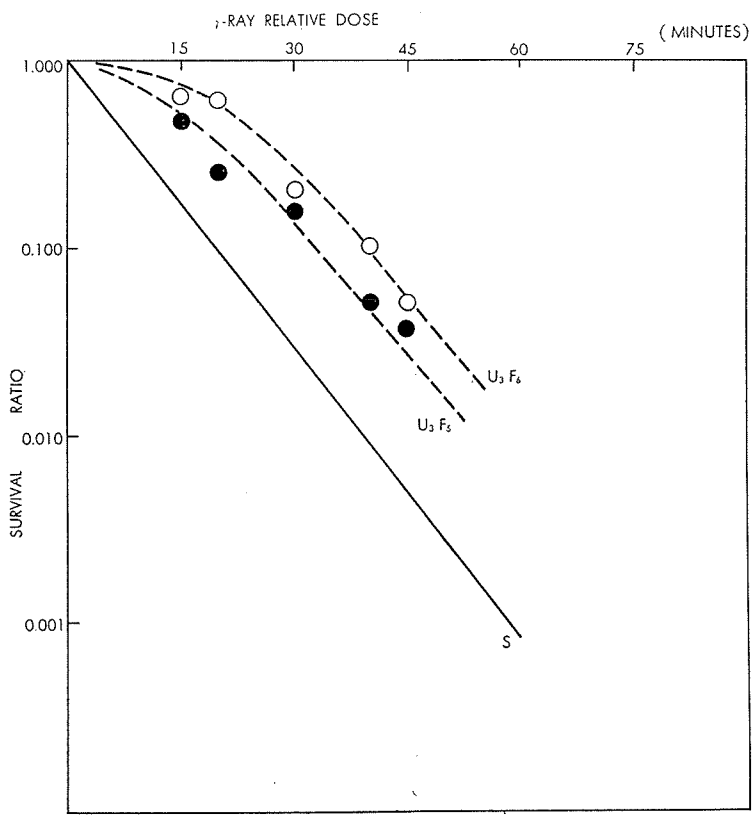

Fig. 6 Survival curves of fifth and sixth fractions obtained by fractionation of a third undiluted passage preparation.

Such a difference might be caused by contamination of the preparations with input virus. To clarify those points, inactivation experiments of infective agents isolated by fractionation described in the preceded section were carried out.

The techniques used are same as in preceded experiments and the samples were also diluted with neutral broth to eliminate the secondary action of radiation.

The fifth and sixth fractions were chosen because the relatively large amounts of the infective agents at the height of the column were the same as those of infective agents in standard passage preparation. In other words if there exist "one hit type" particles in the preparation they should be concentrated in the fifth fraction.

The shoulder in the survival curve of Fig. 6 is unequivocal and gives evidence for a multiple hit inactivation process. This curve for the sixth fraction fits well with

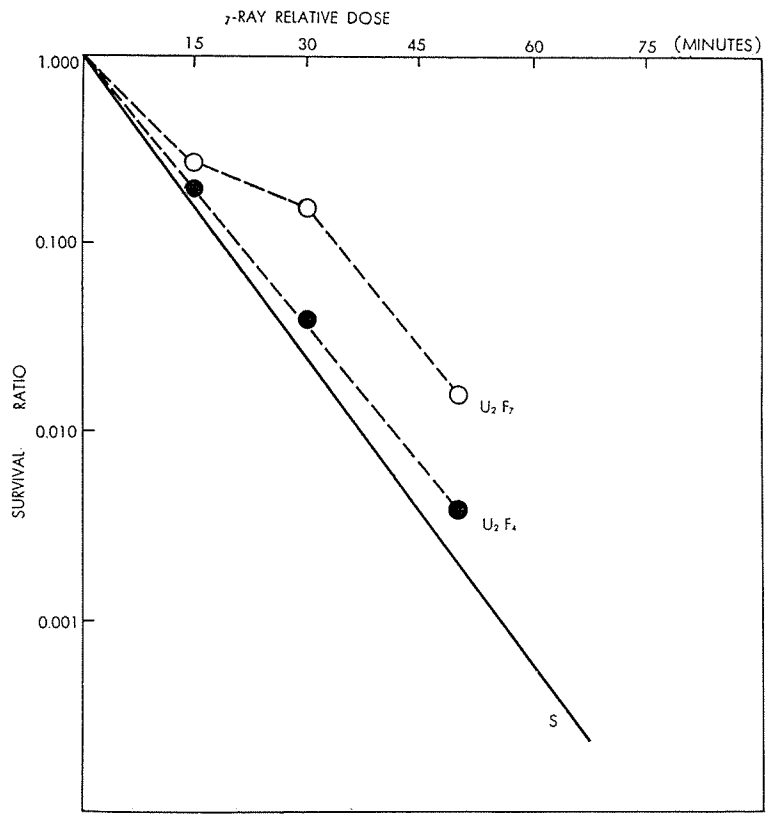

Fig. 7. Survival curves of fourth and seventh fractions obtained by fractionation of a second undiluted passage preparation.

equation $N/N_0 = 1-(1-e^{-\alpha D})^m$ where m is the ionization number required to inactivate one infective agent by the gamma rays. The curve for the fifth fraction still shows contamination with particles which follow the single hit inactivation.

It was observed that there is definitely one faster sedimenting portion in the third undiluted passage preparation and that portion contains special virus particles having the nature of multiple infective centers.

ii) Survival cvrves of fourth and seventh fractions obtained from the second undiluted passage preparation:

In this case two fractions, the fourth and seventh, were chosen for irradiation experiments because from the result of fractionation experiment there may be mixing of rapid and slowly sedimenting infective agents in fifth and sixth fractions. The results are presented in Fig. 7. Slowly sedimenting components in the fourth fraction consist almost entirely of infective agents which follow the single hit curve. On the contrary faster sedimenting components in the seventh fraction were inactivated following multiple hit curve. This agrees with the distribution of the EID_{50} and hemagglutinin in the density gradient fractionation of the second

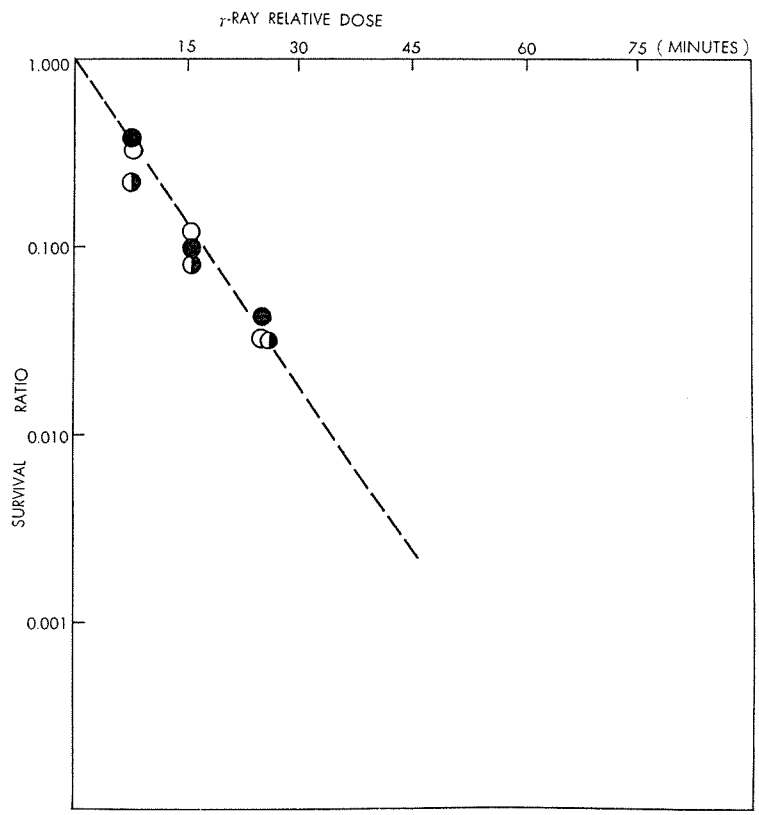

Fig. 8. Survival curves of fifth, sixth, and seventh fractions obtained by fractionation of a standard passage preparation.

◑ Fifth fraction ○ Sixth fraction

● Seventh fraction

undiluted passage preparation. From the distribution curve it can be said that a "single hit" infective agent is present in higher fourth and fifth fractions and the agent in lower sixth and seventh fractions has a different nature.

iii) Survival curves of fifth, sixth, and seventh fractions obtained from the standard passage preparation:

Though the survival curve of the standard passage preparation revealed a single hit type when crude infected chorioallantoic fluid was used in irradiation experiments, there still remains the question of whether there are some "multiple hit" particles in the preparation. Irradiation of the fifth, sixth and seventh fractions obtained from the standard passage preparation showed the same type of single hit survival curve. This excludes the posibility that there are "multiple hit" type particles in the standard passage preparation.

In other words, it can be stated that "multiple hit type" particles do not appear in the standard passage chorioallantoic fluid.

DISCUSSION

The survival curve of PR8 standard passage virus in gamma ray irradiation follows the typical single hit curve. This is another proof that the standard virus preparation consists of homogeneous particles as far as infectivity is concerned.

On the contrary, in irradiation experiments of third undiluted passage chorioallantoic fluid, the situation is quite different; that is, the inactivation curves are very much like to multiple hit type curves. Second undiluted passage chorioallantoic fluid showed a similar curve but it was a transitional type between a standard virus curve and a third undiluted passage virus curve. These two curves shown by second and third undiluted preparations did not fit the theoretical multiple hit curve.

When the preparations were fractionated by the sucrose density gradient method, the results of irradiation experiments were somewhat different. While the inactivation curve of a standard preparation was the same as that of the crude sample in any fraction, the survival curves of individual fractions from the third undiluted preparation fitted well with theoretical multiple hit curves. The latter fact means that there are two kinds of the virus particles the one of which follows a single hit curve inactivation and the other a multiple hit inactivation curve during the irradiation by gamma ray.

Fig. 9 shows synthetic curves from a single hit curve (as in the standard passage preparation) and a multiple hit curve (as in the sixth fraction of a third undiluted passge preparation) with varying proportions of the "single hit type" and "multiple hit type" virus particles.

So far it has been discussed in several reports that an undiluted passage preparation is not homogeneous in size and shape. Besides these findings the author would like to mention that the infective agents in the undiluted passage preparation are not homogeneous from the result of irradiation experiments.

Morphological examination shows many large particles in "multiple hit particle" fractions and filamentous form were observed less frequently. Electron micro-

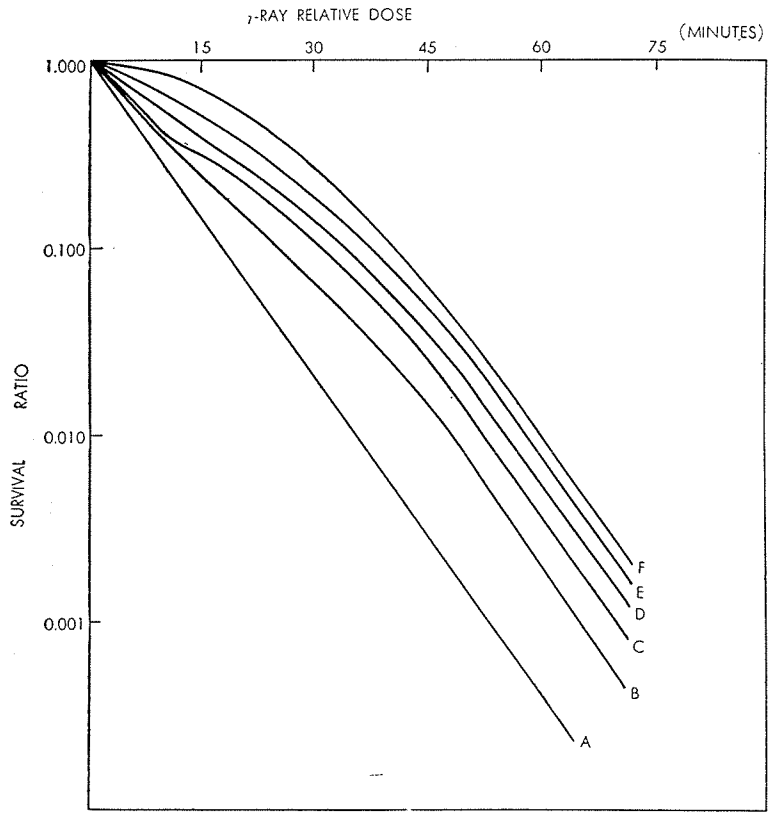

Fig. 9. Survival curves synthesized from a "single hit" curve (survival curve of a standard passage preparation) and "multiple hit" curve (survival curve of a sixth fraction of a third undiluted passage preparation) varying the ratio of the "single hit type" and "multiple hit type" particles.

	"single hit type"	:	"multiple hit type":
A	1.0	:	0.0
B	0.8	:	0.2
C	0.6	:	0.4
D	0.4	:	0.6
E	0.2	:	0.8
F	0.0	:	1.0

graphs of thin sections of the virus pellets of undiluted passage preparations show the existence of electron-dense material inside the particles of the "multiple hit type" while particles in non-infectious hemagglutinating fractions are lacking in that structure. Details of the findings will be reported elsewhere.

The possibility that the inactivation curve in cases of undiluted passage preparations is only apparent and caused by the aggregation of the particles can be neglected because the sucrose density gradient column allows the aggregated par-

ticles to sink to the bottom of the centrifuge tubes.

It is difficult to exclude the possibility of multiplicity reactivation of non-infectious hemagglutinating particles and gamma ray inactivated virus. But, assuming that the chorioallantoic membrane has 2×10^7 cells per egg, there is scarcely a chance of multiple infection in the diluted inoculum used for infectivity titration.

Though other characteristics are shown, it can be emphasized that in undiluted passage preparations of PR8 virus there is a kind of virus particle which is inactivated by gamma ray irradiation following the multiple hit curve and particles of this type can not be found in the standard passage preparation. The author considers that particles of this type have plural infective centers on individual particles.

The observation that the straight line portion of the survival curves of the "multiple hit type" particles is parallel to the inactivation curve of the "single hit type" particles, suggests that the so-called sensitive volume of the infective center is equal in both kinds of the virus particles.

The progeny of virus from all eggs inoculated with sixth fractions of third undiluted passage preparation at limiting dilution was the same as that of a standard passage preparation. Furthermore, the standard virus, sixth fraction of third undiluted passage preparation, and its phylogeny could not be differentiated from each other serologically by the agglutination inhibition test. These facts may suggest that the "multiple hit type" particles belong to a certain stage in virus synthesis, but further speculations must be made with reservation.

ACKNOWLEDGEMENT

The author is grateful to Dr. Konosuke Fukai for his help and guidance throughout the work. Thanks are also due to Miss Kazuko Kawai for help in some of the experiments, and Mr. Yoshimi Nishi for technical assistance.

REFERENCES

Ada, G. L., Perry, B. T. (1954). The nucleic acid content of influenza virus. *Australian J. Exptl. Biol. Med. Sci.* **32**, 453–468.

Lea, D. E. (1947). *Actions of Radiations on Living Cells.* University Press, Cambridge

Mizutani, H. (1958). Studies on the structure of von Magnus type incomplete form of influenza virus I, II, and III. *Virus* 8, 182–201. (in Japanese with English summary).

Mayer, M. M., Rapp, H. J., Roziman, B., Klein, S. W., Cowan, K. M., Lukens, D., Schwerdt, C. E., Schaffer, F. L. and Charney, J. (1957). The purification of poliomyelitis virus as studied by complement fixation. *J. Immunol.* **78**, 435–455.

Shiota, O. (1956). Studies on the incomplete form of influenza virus, II. *Vivus* **6**, 193–206. (in Japanese).

Shiota, O. and Fukazawa, R. (1956). Studies on the incomplete form of influenza virus III. *Virus* **6**, 207–213. (in Japanese).

von Magnus, P (1951). Propagation of the PR8 strain of influenza A virus in chick embryo. II. The formation of incomplete virus following inoculation of large doses of seed virus. *Acta pathologica* **28**, 278–293.

Werner, G. H. and Schlesinger, R. W. (1954). Morphological and quantitative comparison between infectious and non-infectious forms of influenza virus. *J. Exptl. Med.* **100**, 203–216.