# INTERFERENCE BETWEEN THE INFLUENZA VIRUSES

## II. The Effect of Virus Rendered Non-Infective by Ultraviolet Radiation upon the Multiplication of Influenza Viruses in the Chick Embryo*

By JAMES E. ZIEGLER, Jr., M.D.,
*Lieutenant, Medical Corps, United States Naval Reserve*

GEORGE I. LAVIN, Ph.D.,

and FRANK L. HORSFALL, Jr., M.D.,
*Lieutenant Commander, Medical Corps, United States Naval Reserve*

(*From the United States Navy Research Unit at the Hospital of The Rockefeller Institute for Medical Research, and the Laboratories of The Rockefeller Institute for Medical Research*)

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In the preceding paper (1) the effects of active virus upon the multiplication of influenza viruses in the chick embryo were reported, and it was shown that infection of the chick embryo either by influenza A virus, influenza B virus, or by swine influenza virus, produced inhibition of the multiplication of either of the other two viruses subsequently introduced in the embryo.

The work of Delbrück and Luria (2) upon the interference produced by one strain of *Escherichia coli* bacteriophage with the multiplication of another strain active against the same microorganism yielded results which have certain similarities with the results obtained with the influenza viruses. In a subsequent paper Luria and Delbrück (3) reported the effect upon the interference phenomenon of the inactivation of bacteriophage by ultraviolet light. They showed that ultraviolet irradiation could destroy the properties of multiplication and bacteriolysis of bacteriophage, without apparent impairment of its ability to produce bacteriostasis or to produce interference with the multiplication of another strain of bacteriophage. Salk, Lavin, and Francis (4) have shown that influenza virus could be inactivated by exposure to ultraviolet radiation and that such preparations retained the capacity to immunize mice.

The present paper reports observations on the effects upon the multiplication in the chick embryo of influenza A virus, influenza B virus, or swine influenza virus which were produced by the presence of influenza A or influenza B virus rendered non-infective by ultraviolet radiation.

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### *Methods*

The materials and methods used in this study were identical in all respects to those described in the preceding paper (1).

## EXPERIMENTAL

*Effect of Exposure to Ultraviolet Radiation upon the Infectivity of Influenza A and Influenza B Viruses.*—Allantoic fluid containing either influenza A virus (PR8 strain) or influenza B virus (Lee strain ) was exposed to ultraviolet radiation for varying periods of time, and the irradiated preparations were tested for infectivity.

Allantoic fluid pools containing either active PR8 or Lee virus, with from 1 to 5 per cent of normal horse serum added, were exposed in 50 cc. quartz test tubes to ultraviolet radiation using the apparatus and method described by Havens *et al.* (5). The source of the radiation was a spirally coiled quartz Hg resonance lamp, from which approximately 85 per cent of the energy was emitted as the 2537Å line. The quartz tube containing the specimen was suspended in the center of the spiral lamp, the coils of which extended above and below the fluid in the tube. Samples of the irradiated fluid were withdrawn after various periods of exposure. These were stored at 4°C.

The infectivity of the samples was tested in the chick embryo. Each sample was inoculated undiluted into a group of 4 to 6 normal embryos. After incubating for 48 hours the allantoic fluids were removed and the presence or absence of RBC agglutination was noted. When RBC agglutination was not produced, these allantoic fluids were passed to a second group of normal embryos which were then treated in a similar manner. Certain samples were also tested by intranasal inoculation in white Swiss mice.

It was found that continued exposure to ultraviolet radiation progressively inactivated both PR8 and Lee viruses. Following relatively short periods of irradiation, allantoic fluids from embryos inoculated with the irradiated preparations failed to cause RBC agglutination. The passage of such allantoic fluids to a second group of test embryos, however, frequently revealed that small amounts of active virus were, in fact, present since RBC agglutination was produced by the allantoic fluids of the second passage embryos. Irradiated pools which failed to produce evidence of infection on first embryo passage, but caused manifest infection on a second embryo passage, were termed "partially inactivated."

After longer periods of irradiation, not only was RBC agglutination not demonstrable with the allantoic fluids obtained from the first passage embryos, but also it was not demonstrable with allantoic fluids from the second or even from the third passage embryos. In no instance did active virus become demonstrable in the third serial embryo passage when RBC agglutination was not observed with the allantoic fluids from the second embryo passage. Since serial passage in chick embryos is probably as delicate a method as any yet

devised for the detection of small quantities of active influenza virus, it was considered that irradiated pools which failed to produce evidence of infection on 2 embryo passages contained only "non-infective" virus. Such irradiated pools were found to produce no evidence of infection when inoculated intranasally in Swiss mice.

*The Effect of Partially Inactivated Lee Virus upon the Multiplication of Active PR8 or Lee Virus.*—The effect of partial inactivation by ultraviolet radiation upon the interference produced by Lee virus was investigated.

The methods used in testing the capacity of ultraviolet irradiated virus to produce interference were similar to those used in previous experiments with active virus.

A number of normal chick embryos were each inoculated with 0.1 cc. of undiluted allantoic fluid containing Lee virus, which had been exposed to ultraviolet radiation. This allantoic fluid failed to produce manifest infection of chick embryos on first passage, although on second passage RBC agglutination occurred. 24 hours after inoculation with this allantoic fluid groups of embryos were reinoculated with $10^4$, $10^5$, or $10^6$ E.I.D., respectively, of PR8 virus, while other groups were reinoculated with $10^4$, $10^5$, or $10^6$ E.I.D., respectively, of Lee virus. At the same time, normal embryos were inoculated with the active viruses. All the embryos were then incubated for 48 hours after which the allantoic fluids were removed and the presence or absence of RBC agglutination was noted.

The results are shown in Table I. All of the control embryos inoculated with active virus yielded allantoic fluids which gave strong RBC agglutination, while none of the embryos inoculated only with the partially inactivated virus gave allantoic fluids which agglutinated RBC. With but one exception, all of the embryos inoculated initially with partially inactivated Lee virus and subsequently with large amounts of active PR8 or Lee virus, yielded allantoic fluids which did not agglutinate RBC. The single exception was an allantoic fluid which caused only slight agglutination. This fluid was obtained from an embryo which had been reinoculated with $10^6$ E.I.D. of PR8 virus.

It seems apparent that partially inactivated Lee virus, in an amount which represented approximately $10^7$ E.I.D. before exposure to ultraviolet radiation, was capable of inhibiting the multiplication in the chick embryo of very large amounts of either active PR8 or Lee virus subsequently introduced.

*The Effect of PR8 or Lee Virus, Rendered Non-Infective by Ultraviolet Radiation, upon the Multiplication of Influenza Viruses in the Chick Embryo.*—Allantoic fluid pools containing PR8 or Lee virus, which had been exposed to ultraviolet radiation and shown to be non-infective for mice and for chick embryos on serial passage, were tested to determine their capacity to produce interference.

This experiment was carried out in the same manner as the preceding experiment with partially inactivated virus. A number of embryos were each inoculated with

0.1 cc. of undiluted allantoic fluid which contained either PR8 or Lee virus made completely non-infective for mice and for chick embryos by ultraviolet radiation. After 24 hours' incubation groups of embryos which had received non-infective PR8 virus were reinoculated with 10 E.I.D. of PR8, Lee, or swine virus, respectively, while groups of embryos which had been given non-infective Lee virus were reinoculated with 10, $10^2$, or $10^3$ E.I.D. of PR8 virus, 10 or $10^2$ E.I.D. of Lee virus, or 10 E.I.D.

TABLE I

*Effect of Lee Virus Partially Inactivated by Ultraviolet Radiation upon Multiplication in the Chick Embryo of Large Inocula of Active Lee or PR8 Viruses*

| Initial inoculum | Second inoculum 24 hrs. after first inoculation | RBC agglutination (48 hrs. after second inoculation) |
|---|---|---|
| | *E.I.D.* | |
| Undiluted allantoic fluid (Lee) partially inactivated by ultraviolet | PR8 $10^4$ | 0/4* |
| | " $10^5$ | 0/4 |
| | " $10^6$ | 1/5 |
| | Lee $10^4$ | 0/4 |
| | " $10^5$ | 0/5 |
| | " $10^6$ | 0/4 |
| None (controls) | PR8 $10^4$ | 3/3 |
| | " $10^6$ | 6/6 |
| | Lee $10^4$ | 3/3 |
| | " $10^6$ | 6/6 |
| Undiluted allantoic fluid (Lee) partially inactivated by ultraviolet | None | 0/6‡ |

* The numerator represents the number of allantoic fluids which produced RBC agglutination. The denominator indicates the number of embryos inoculated.

‡ On second passage these allantoic fluids produced infection of the chick embryo.

of swine virus, respectively. After an additional 48 hours' incubation the individual allantoic fluids were removed and tested.

The results of these experiments can be seen in Table II. None of the allantoic fluids removed from embryos initially inoculated with non-infective PR8 virus, followed by active Lee virus, produced RBC agglutination, and only 1 of 8 fluids from embryos inoculated with non-infective PR8 virus followed by active PR8 virus produced RBC agglutination. However, of 4 embryos inoculated with non-infective PR8 virus followed by active swine virus, 3 yielded allantoic fluids which gave RBC agglutination. The allantoic fluids from embryos initially inoculated with non-infective Lee virus and subsequently reinoculated with active PR8, Lee, or swine virus produced RBC agglutination in only 2 instances. One of 4 embryos reinoculated with $10^3$ E.I.D. of PR8

virus yielded an agglutinating allantoic fluid, as did 1 of 7 embryos reinoculated with 10 E.I.D. of swine virus. The control embryos, inoculated only with active virus, yielded allantoic fluids which, with but 2 exceptions, produced strong RBC agglutination.

These results indicate that ultraviolet-irradiated influenza virus preparations in which the property of multiplication appeared to have been completely

TABLE II

*Effect of PR8 or Lee Virus Made Non-Infective by Ultraviolet Radiation upon Multiplication of Active Influenza Viruses in the Chick Embryo*

| Initial inoculum (0.1 cc.) | Second inoculum 24 hrs. after first inoculation | RBC agglutination (48 hrs. after second inoculation) |
|---|---|---|
| | *E.I.D.* | |
| Undiluted non-infective allantoic fluid (PR8) | PR8 10 | 1/8* |
| | Lee 10 | 0/6 |
| | Swine 10 | 3/4 |
| | None | 0/6‡ |
| Undiluted non-infective allantoic fluid (Lee) | PR8 10 | 0/10 |
| | " $10^2$ | 0/4 |
| | " $10^3$ | 1/4 |
| | Lee 10 | 0/10 |
| | " $10^2$ | 0/8 |
| | Swine 10 | 1/7 |
| | None | 0/6‡ |
| None (controls) | PR8 10 | 9/10 |
| | " $10^2$ | 4/4 |
| | " $10^3$ | 4/4 |
| | Lee 10 | 10/10 |
| | " $10^2$ | 8/8 |
| | Swine 10 | 9/10 |

* The numerator represents the number of allantoic fluids which produced RBC agglutination. The denominator indicates the number of embryos inoculated.

‡ These allantoic fluids failed to produce infection on 3 serial embryo passages.

destroyed and which failed to produce infection in mice or on serial chick embryo passage were still capable of producing interference with the multiplication of subsequently inoculated influenza virus. Except in the case of embryos prepared with non-infective PR8 virus and tested with swine virus, it is of interest that interference was produced by these non-infective preparations whether the test virus was the same or a wholly different virus strain. The capacity of these non-infective allantoic fluids to produce interference remained unimpaired during storage at 4°C. for 4 weeks.

*Effect of Non-Infective Virus upon Multiplication of Active Virus When Both*

*Were Inoculated Simultaneously in Chick Embryos.*—In the light of the preceding experiments with non-infective virus it seemed possible that the absence of RBC agglutination after a single embryo passage of virus rendered partially inactive by ultraviolet radiation might be explained on the basis of "auto-interference"; that is, by the inhibition of the multiplication of residual active virus which resulted from the presence of a large amount of non-infective virus in the same preparation. This assumption could be tested by adding active virus to a non-infective virus preparation and determining whether or not the former virus multiplied after inoculation of chick embryos with the mixture.

Active Lee virus was added to an allantoic fluid containing Lee virus made non-infective by ultraviolet radiation. Normal embryos were inoculated with 0.1 cc. of this mixture. The inoculum contained $10^2$ E.I.D. of active Lee virus and an amount of non-infective virus which before ultraviolet irradiation approximated $10^6$ E.I.D. The embryos were incubated for 48 hours, after which the allantoic fluids were removed individually, and the presence or absence of RBC agglutination was noted. A group of control embryos inoculated with the same dilution of active Lee virus was treated similarly.

None of the allantoic fluids from embryos inoculated with a mixture of active Lee virus and non-infective Lee virus produced RBC agglutination. On the other hand, all of the control embryos inoculated with the same amount of active Lee virus yielded allantoic fluids which produced strong RBC agglutination. These results indicate that non-infective virus inhibited the multiplication of active virus when both were introduced simultaneously into chick embryos.

*Attempts to Demonstrate a Tranmissible Substance Responsible for the Interference Phenomenon.*—The appearance of RBC agglutination after a second embryo passage of partially inactivated virus preparations made it seem probable that the capacity of non-infective virus to produce interference was not caused by a transmissible agent. This possibility was tested directly by attempting to use as interfering agents allantoic fluids from embryos in which the multiplication of active PR8, Lee, or swine virus had been inhibited by a non-infective Lee virus preparation, as well as allantoic fluids from embryos which had received only non-infective Lee virus.

Allantoic fluids from embryos which had been initially inoculated with a non-infective Lee virus allantoic fluid, followed in 24 hours by reinoculation with 10 or $10^2$ E.I.D. of active PR8, Lee, or swine virus, respectively, were shown to produce no RBC agglutination. Groups of chick embryos were inoculated with 0.1 cc. of these allantoic fluids. 24 hours later they were reinoculated with $10^2$ E.I.D. of active Lee virus. After 48 hours' further incubation the allantoic fluids were removed and tested for RBC agglutination, as well as for the identity of the virus present. In a second experiment, allantoic fluids harvested 48 hours after inoculation of the embryos with 0.1 cc. of non-infective Lee virus allantoic fluid were pooled and used as the initial

inoculum. 24 hours later the subinoculated embryos were reinoculated with $10^2$ E.I.D. of Lee virus and treated as described above.

The results of these experiments were unequivocal. In every instance a second embryo passage of non-infective virus failed to produce any evidence of interference. Reinoculation of embryos, which had received the first passage material, with active Lee virus resulted in manifest infection, and the RBC agglutination which occurred was completely inhibited by anti-Lee virus rabbit serum.

Under the conditions of these experiments it seems evident that the agent responsible for the interference produced by non-infective Lee virus preparations was not transmissible from embryo to embryo.

*Attempts to Demonstrate Active Virus in Chick Embryos after Multiplication Had Been Inhibited by Either Partially Inactivated or Non-Infective Virus.*—The demonstration of interference with the multiplication of active influenza virus by non-infective influenza virus in the chick embryo and the finding that the property of interference was not transmissible in series provided means of determining more precisely the fate of the active virus, the multiplication of which was inhibited.

A number of embryos were given an initial inoculum of 0.1 cc. of non-infective Lee virus allantoic fluid. Subsequently groups of these embryos were reinoculated with $10^2$ E.I.D. of active PR8, Lee, or swine virus, respectively. After 48 hours' incubation the allantoic fluids obtained from all these embryos failed to agglutinate RBC. 0.1 cc. of each of these allantoic fluids was then inoculated in 2 normal chick embryos which were subsequently incubated for 48 hours. The allantoic fluids from these subinoculated embryos were then removed individually and tested for RBC agglutination. When agglutination occurred the virus present was identified by the method described above.

It was found that each of the allantoic fluids obtained from embryos inoculated initially with non-infective Lee virus and subsequently with either active PR8 or Lee virus, on subinoculation in normal embryos gave allantoic fluids which failed to agglutinate RBC. Of the allantoic fluids from the embryos inoculated initially with non-infective Lee virus and subsequently with active swine virus, 2 on subinoculation in normal embryos gave rise to allantoic fluids which failed to agglutinate RBC, while the remaining 2 caused the development of RBC agglutination. Anti-swine virus rabbit serum completely inhibited the agglutination caused by these latter fluids.

These results indicate that, as was the case with interference produced by active virus, non-infective Lee virus was effective in inhibiting the multiplication of small inocula of either active PR8 or active Lee virus, and no evidence was obtained that multiplication of the active virus had occurred. However,

under the same conditions the multiplication of swine virus was not completely inhibited in all instances.

A similar experiment was carried out with allantoic fluids obtained from embryos in which it was shown that Lee virus partially inactivated by ultraviolet radiation had inhibited the multiplication of large inocula of either active PR8 or Lee virus.

Allantoic fluids obtained from groups of embryos in which partially inactivated Lee virus had inhibited the multiplication of an inoculum of (1) $10^5$ E.I.D. of active PR8 virus, (2) $10^6$ E.I.D. of active RP8 virus, or (3) $10^6$ E.I.D. of active Lee virus, were used. Allantoic fluids from 2 embryos in each of these 3 groups were tested in normal embryos. Each allantoic fluid was inoculated undiluted, as well as in tenfold

TABLE III

*Effect of Lee Virus Partially Inactivated by Ultraviolet Radiation upon Multiplication of Large Inocula of Active Influenza Viruses in the Chick Embryo*

| Initial inoculum (0.1 cc.) | Second inoculum 24 hrs. after first inoculation | RBC agglutination (48 hrs. after second inoculation) | Embryo infectivity titer |
|---|---|---|---|
| | *E.I.D.* | | |
| Undiluted allantoic fluid (Lee) partially inactivated by ultraviolet | PR8 $10^5$ | 0 | $10^{-3}$ |
| | " $10^5$ | 0 | $10^{-4}$ |
| | " $10^6$ | 0 | $<10^{-1}$ |
| | " $10^6$ | 0 | $10^{-1}$ |
| | Lee $10^6$ | 0 | $10^{-1}$ |
| | " $10^6$ | 0 | $10^{-1}$ |

dilutions in sterile broth from $10^{-1}$ to $10^{-4}$. The inoculated embryos were incubated for 48 hours, after which the allantoic fluids were removed and tested for RBC agglutination. The virus present was identified as described above.

The results of this experiment are shown in Table III. Allantoic fluids from embryos initially inoculated with partially inactivated Lee virus and subsequently reinoculated with $10^6$ E.I.D. of active PR8 or Lee virus were found to have embryo infectivity titers of only $10^{-1}$. In each instance RBC agglutination was inhibited by antiserum against the active virus used as the second inoculum. However, the allantoic fluids from embryos inoculated with partially inactivated Lee virus, and reinoculated with $10^5$ E.I.D. of active PR8 virus were found to have embryo infectivity titers of $10^{-3}$ and $10^{-4}$, respectively. These results indicate that the inhibition of the multiplication of large inocula of either active PR8 or Lee virus by partially inactivated Lee virus was practically complete in most instances, even though some active virus persisted in the embryo. The observed titers were, in 4 instances, not as high as would have been expected had all the active virus inoculated remained unaltered in the

embryo and undergone no multiplication whatsoever. In 2 instances the observed titers were slightly higher than could be accounted for on the basis of dilution in the embryo and in the extra-embryonic fluids. However, even in these 2 instances the apparent multiplication was no greater than fivefold and 50-fold, respectively.

*The Effect of Prolonged Ultraviolet Irradiation of PR8 or of Lee Virus upon the Interference Phenomenon.*—In view of the results of the preceding experiments, which indicated that influenza virus rendered non-infective by ultraviolet radiation retained the capacity to cause interference, it was of interest to investigate the effects of even more prolonged irradiation upon the virus.

Irradiated samples from 3 separate allantoic fluid pools containing influenza virus were tested for their capacity to produce interference. Specimens from a pool containing PR8 virus which were removed after ultraviolet irradiation for 30, 40, and 50 minutes, respectively, were tested. Specimens from a pool containing Lee virus which were withdrawn after ultraviolet irradiation for 15, 20 and 30 minutes, respectively, as well as specimens from another Lee virus pool removed after 25 and 30 minutes' exposure, respectively, were also tested. Each of these specimens failed to produce any evidence of infection in the chick embryo on serial passage. Other samples removed from each of these 3 pools after shorter periods of irradiation were found to have been only partially inactivated and by serial embryo passage the presence of some active virus was demonstrated in each sample. Groups of embryos were inoculated with each of the specimens indicated above, and 24 hours later one-half of each group were reinoculated with $10^2$ E.I.D. of Lee virus while the remainder were reinoculated with $10^2$ E.I.D. of PR8 virus. Simultaneously the active viruses were also inoculated in normal embryos. After 48 hours' incubation the allantoic fluids removed from these embryos were tested for RBC agglutination.

The results of this experiment appear in Table IV. When PR8 virus irradiated for 30 minutes was used as the initial inoculum, none of the embryos reinoculated with either PR8 or Lee virus yielded allantoic fluids which agglutinated RBC. When a sample of the same pool irradiated for 40 minutes was tested similarly none of 3 embryos reinoculated with Lee virus produced RBC agglutinating allantoic fluids, but 1 of 3 embryos reinoculated with PR8 virus did so. Finally, when a sample of the same pool exposed to ultraviolet light for 50 minutes was used as the initial inoculum, the allantoic fluid from 1 of 3 eggs reinoculated with Lee virus produced RBC agglutination, and all of the allantoic fluids from embryos reinoculated with PR8 virus did so.

Embryos initially inoculated with Lee virus which had been irradiated for 25 minutes, and reinoculated with either PR8 or Lee virus, yielded allantoic fluids of which none produced RBC agglutination. No RBC agglutination was produced by the allantoic fluids of embryos which received Lee virus after inoculation with a sample of the same pool irradiated for 30 minutes. However, 2 of 4 embryos inoculated with this sample, and reinoculated with PR8 virus, gave allantoic fluids which produced RBC agglutination.

Samples from another Lee virus pool gave similar results. When a sample irradiated for 15 minutes was used as the initial inoculum, none of the embryos reinoculated with Lee virus yielded allantoic fluids which produced RBC agglutination, although 1 of 4 embryos reinoculated with PR8 virus did so.

TABLE IV

*Effect of Prolonged Ultraviolet Irradiation of PR8 and Lee Viruses upon Their Capacity to Produce Interference with the Homologous and Heterologous Virus*

| Initial inoculum 0.1 cc. undiluted allantoic fluid | | Second inoculum 24 hrs. after first inoculation | RBC agglutination (48 hrs. after second inoculation) |
|---|---|---|---|
| Allantoic fluid pool* | Exposed to ultraviolet radiation | | |
| | *min.* | $10^3$ *E.I.D.* | |
| PR8 | 30 | Lee | 0/4‡ |
| | | PR8 | 0/4 |
| | 40 | Lee | 0/3 |
| | | PR8 | 1/3 |
| | 50 | Lee | 1/3 |
| | | PR8 | 4/4 |
| Lee | 25 | Lee | 0/4 |
| | | PR8 | 0/4 |
| | 30 | Lee | 0/4 |
| | | PR8 | 2/4 |
| Lee | 15 | Lee | 0/4 |
| | | PR8 | 1/4 |
| | 20 | Lee | 1/4 |
| | | PR8 | 4/4 |
| | 30 | Lee | 3/3 |
| | | PR8 | 3/3 |
| None | — | Lee | 14/14 |
| | | PR8 | 13/14 |

* Each of the samples of irradiated allantoic fluid pools used in these tests was shown to be non-infective on serial embryo passage.

‡ The numerator represents the number of allantoic fluids which produced RBC agglutination. The denominator indicates the number of embryos inoculated.

When a sample irradiated for 20 minutes was tested, 1 of 4 embryos reinoculated with Lee virus and all reinoculated with PR8 virus gave allantoic fluids which caused RBC agglutination. When a similar test was carried out with a sample irradiated for 30 minutes, no evidence of interference with the multiplication of either virus was obtained.

These results indicate that with increasing periods of exposure to ultraviolet radiation the capacity of irradiated allantoic fluids to produce interference progressively decreased and finally was abolished.

It was observed that the capacity of allantoic fluids containing influenza virus to interfere with PR8 virus was destroyed by prolonged ultraviolet irradiation before the capacity to interfere with Lee virus was lost, in all preparations in which it was possible to carry out the necessary tests. In some instances, for reasons which are not entirely clear, the interval between the time of exposure to ultraviolet radiation which caused loss of infectivity, and the time of exposure which caused loss of the capacity to produce interference, was so short as to make it impossible to determine this point.

*Effect of Dilution of Non-Infective Lee Virus upon Its Capacity to Produce Interference.*—In the preceding experiments with influenza viruses rendered non-infective by ultraviolet radiation only undiluted allantoic fluids were used in interference tests. It seemed desirable to determine the extent to which non-infective allantoic fluid could be diluted and still retain the capacity to interfere with the multiplication of influenza viruses in chick embryos.

An allantoic fluid pool containing Lee virus, which had been exposed to ultraviolet radiation until the virus became non-infective, was used. This specimen was shown in earlier experiments to be capable of causing interference. Serial twofold dilutions in sterile broth were made. Groups of embryos were inoculated with 0.1 cc. of each of the dilutions, and 24 hours later half of each group were reinoculated with 10 E.I.D. of PR8 virus, while the other half were reinoculated with 10 E.I.D. of Lee virus. The embryos were then incubated for 48 hours and their allantoic fluids tested subsequently in the usual manner.

The results of this experiment are seen in Table V. When the undiluted allantoic fluid pool was inoculated in embryos which were later reinoculated with either PR8 or Lee virus, all of the embryos gave allantoic fluids which failed to agglutinate RBC. Embryos inoculated with a 1:2 dilution of the pool, and reinoculated with PR8 virus, gave allantoic fluids of which only 1 of 4 produced RBC agglutination, while of those reinoculated with Lee virus none produced RBC agglutinating fluids. When a 1:4 or a 1:8 dilution of the pool was used, embryos reinoculated with either Lee or PR8 virus yielded allantoic fluids of which all but 2 produced RBC agglutination.

These results indicate that allantoic fluid containing non-infective Lee virus lost the capacity to produce interference on slight dilution, and suggest that very large amounts of non-infective virus were required to cause interference. Since there was no significant difference between the degree of interference produced against either the homologous Lee virus or the heterologous PR8 virus at any dilution tested, it seems evident that quantitative factors alone do not explain the differences which were observed in the preceding experiments when specimens which had been subjected to prolonged irradiation were tested against both these viruses.

The results of this experiment with dilutions of non-infective virus led to

additional tests with dilutions of partially inactivated Lee virus. It was found that although the undiluted preparation of partially inactivated virus failed to produce RBC agglutinating allantoic fluids on first embryo passage, a second passage resulted in manifest infection. When, however, a $10^{-1}$ dilution of this preparation was inoculated, RBC agglutinating allantoic fluids were produced by the first passage embryos. This indicates that the "auto-interference" demonstrable with partially inactivated virus may be abolished by dilution. Moreover, these results suggest a method for testing for the presence of active virus more rapid than that of serial embryo passage. In the light of the pre-

TABLE V

*Effect of Dilution of Non-Infective Lee Virus upon Its Capacity to Produce Interference*

| Initial inoculum 0.1 cc. u.v. irradiated non-infective Lee virus allantoic fluid | Second inoculum 24 hrs. after first inoculation | RBC agglutination by allantoic fluids (48 hrs. after second inoculation) |
|---|---|---|
| *dilution* | *10 E.I.D.* | |
| None | PR8 | 0/4* |
| | Lee | 0/4 |
| 1:2 | PR8 | 1/4 |
| | Lee | 0/4 |
| 1:4 | PR8 | 3/4 |
| | Lee | 4/4 |
| 1:8 | PR8 | 3/4 |
| | Lee | 4/4 |
| No initial inoculation | PR8 | 4/4 |
| | Lee | 4/4 |

* The numerator represents the number of allantoic fluids which produced agglutination. The denominator represents the number of embryos inoculated.

ceding experiments it would be expected that $10^{-1}$ dilutions of partially inactivated virus which contained more than 10 E.I.D. of active virus per 0.1 cc. should cause manifest infection of the embryo even though the undiluted preparation would not do so.

*Attempts to Separate from the Virus the Agent Responsible for Interference.*— Although it seemed logical to assume that the capacity of non-infective virus preparations to produce interference was attributable to the altered virus itself, several experiments were carried out in attempts to determine whether or not the virus and the agent responsible for interference could be separated. In one experiment, the active virus was removed by filtration from a partially inactivated allantoic fluid pool, and the filtrate was tested for its capacity to produce interference.

An allantoic fluid pool containing Lee virus and 1 per cent of normal horse serum was exposed to ultraviolet radiation for 40 minutes, without completely destroying its infectivity. This pool was then passed through a Seitz filter with a double thickness pad. The filtrate caused only questionable RBC agglutination and failed to produce any evidence of infection in the chick embryo. The filtrate was tested in the usual manner for its capacity to produce interference. 10 E.I.D. of either PR8 or Lee virus were used as the active virus inoculum.

All of the embryos inoculated initially with this Seitz filtrate and subsequently inoculated with either PR8 or Lee virus yielded allantoic fluids which produced RBC agglutination.

In another experiment Lee virus rendered non-infective by ultraviolet radiation was inoculated in embryos previously treated with homologous antiserum, and subsequently tested for its capacity to produce interference.

Five groups of chick embryos were inoculated in the following manner: Group 1 was inoculated with 0.1 cc. of non-infective Lee virus allantoic fluid and reinoculated 24 hours later with $10^3$ E.I.D. of PR8 virus. Groups 2 and 3 received 0.1 cc. of a 1:25 dilution of anti-Lee virus rabbit serum in saline solution, and groups 4 and 5 received 0.1 cc. of a similar dilution of anti-PR8 virus rabbit serum. These sera were obtained from rabbits 13 days after a single intravenous injection of PR8 or Lee virus concentrated from allantoic fluid by adsorption on chick RBC and subsequent elution into saline solution, as described by Francis and Salk (6). Group 2 was reinoculated 5 hours after the antiserum was given with 0.1 cc. of non-infective Lee virus allantoic fluid and 24 hours later with $10^3$ E.I.D. of PR8 virus. Group 3 was reinoculated 24 hours after the antiserum was injected with a similar amount of PR8 virus. Group 4 was reinoculated 5 hours after receiving the PR8 antiserum with 0.1 cc. of non-infective Lee virus allantoic fluid and 24 hours later with $10^3$ E.I.D. of Lee virus. Group 5 was reinoculated 24 hours after the PR8 antiserum was injected, with a similar amount of Lee virus. The embryos were incubated for 48 hours following the inoculation of active virus. The allantoic fluids were then removed and the presence or absence of RBC agglutination was noted.

The results of this experiment are shown in Table VI. Of 9 embryos inoculated with non-infective Lee virus followed by active PR8 virus, only 1 yielded allantoic fluid which produced RBC agglutination. Of 8 embryos similarly treated but with the addition of anti-Lee virus serum, 6 produced RBC agglutinating allantoic fluids. Of 7 embryos inoculated with anti-Lee virus serum followed by active PR8 virus, 6 produced RBC agglutinating allantoic fluids. Of 6 embryos inoculated with anti-PR8 virus serum followed by non-infective Lee virus and later by active Lee virus, none yielded allantoic fluid which produced RBC agglutination. All of 6 embryos similarly treated, but without the addition of non-infective Lee virus, produced allantoic fluids which caused RBC agglutination.

The results of these experiments indicate that filtration of an irradiated Lee

virus allantoic fluid pool through a Seitz pad not only removed the infective virus, but also removed the agent responsible for interference. Furthermore, they indicate that anti-Lee virus rabbit serum, capable of specifically neutralizing the homologous virus but not the heterologous virus, also neutralized the capacity of non-infective Lee virus to produce interference. As well, they indicate that such neutralization was mediated by antibody directed against Lee virus itself, since serum from a rabbit injected with similar amounts of concentrated PR8 virus failed to neutralize the effect of the non-infective Lee preparation. These experiments suggest that the altered virus itself was the agent responsible for the production of interference.

TABLE VI

*Effect of Specific Rabbit Antiserum on the Capacity of Ultraviolet Irradiated Non-Infective Lee Virus to Produce Interference*

| Preliminary serum inoculation | Initial virus inoculum 5 hrs. after preliminary inoculation | Second virus inoculum 24 hrs. after first inoculation | RBC agglutination by allantoic fluids 48 hrs. after second inoculation |
|---|---|---|---|
| | | $10^8$ *E.I.D.* | |
| None | Non-infective Lee virus, 0.1 cc. undiluted allantoic fluid | PR8 | 1/9* |
| 0.1 cc. anti-Lee virus rabbit serum 1:25 | " " | PR8 | 6/8 |
| " " | None | PR8 | 6/7 |
| 0.1 cc. anti-PR8 virus rabbit serum 1:25 | Non-infective Lee virus, 0.1 cc. undiluted allantoic fluid | Lee | 0/6 |
| " " | None | Lee | 6/6 |

* The numerator represents the number of allantoic fluids which produced RBC agglutination. The denominator indicates the number of embryos inoculated.

*Effect upon the Multiplication of Active Virus in the Embryo of the Subsequent Inoculation of Non-Infective Virus.*—In preceding experiments it was shown that non-infective virus interfered with the multiplication of active virus when it was introduced into the embryo before or simultaneously with the inoculation of active virus. It was shown as well that the interference produced by non-infective virus appeared not to depend on its multiplication in the embryo. An experiment was carried out to determine whether non-infective virus was capable of causing interference when introduced into the embryo after the inoculation of active virus.

Four groups of embryos were inoculated with $10^2$ E.I.D. of Lee virus. After 4 hours' incubation one-half of the first group (1*b*) were reinoculated with 0.1 cc. of Lee virus allantoic fluid made non-infective by ultraviolet radiation. At the same time the allantoic fluids were removed from the remaining embryos of this group (1*a*).

The virus titer of these fluids, after pooling, was determined in chick embryos. The second and third groups were treated in a similar manner 8 hours (groups 2*a* and 2*b*) and 12 hours (groups 3*a* and 3*b*), respectively, after the initial inoculation with active virus. Group 4 received no second inoculation and served as controls. All of the embryos reinoculated with non-infective virus (groups 1*b*, 2*b*, and 3*b*), as well as the control embryos, were incubated for a total of 48 hours following the initial inoculation. The allantoic fluids were then removed individually and tested for RBC agglutination. At the same time the pooled allantoic fluids from each group were titered in chick embryos.

TABLE VII

*Effect upon the Multiplication in the Chick Embryo of Lee Virus of the Subsequent Inoculation of Non-Infective Lee Virus*

| Embryo group | Initial inoculum | Interval between initial inoculation and reinoculation with 0.1 cc. non-infective irradiated Lee allantoic fluid, undiluted | Total period of incubation after inoculation | RBC agglutination | Virus titer |
|---|---|---|---|---|---|
| | | | | Of allantoic fluids, at indicated time of incubation | |
| | *E.I.D.* | | *hrs.* | | |
| 1 *a* | Lee $10^2$ | Not reinoculated | 4 | 0/4* | $10^{-1}$ |
| 1 *b* | " $10^2$ | 4 hrs. | 48 | 0/4 | $10^{-1}$ |
| 2 *a* | " $10^2$ | Not reinoculated | 8 | 0/4 | $10^{-1}$ |
| 2 *b* | " $10^2$ | 8 hrs. | 48 | ±/4 | $10^{-2}$ |
| 3 *a* | " $10^2$ | Not reinoculated | 12 | 0/4 | 0 |
| 3 *b* | " $10^2$ | 12 hrs. | 48 | 0/1 | $10^{-1}$ |
| 4 | " $10^2$ | Not reinoculated | 48 | 5/5 | $10^{-7}$ |

* The numerator represents the number of allantoic fluids which produced RBC agglutination. The denominator indicates the number of embryos inoculated.

The results of this experiment are shown in Table VII. None of the embryos which received non-infective Lee virus 4, 8, or 12 hours after inoculation with active Lee virus yielded allantoic fluids which produced RBC agglutination. All of the allantoic fluids from the control embryos, which were inoculated only with active virus, produced strong RBC agglutination.

The virus titers of the allantoic fluids obtained 4, 8, or 12 hours after inoculation with active virus were not significantly different from the virus titers of the fluids obtained from embryos which were reinoculated with non-infective virus at the same intervals after initial infection and which were incubated for a total of 48 hours. It is notable, as well, that these titers were far below the titer of the pooled allantoic fluids obtained from control embryos inoculated only with active virus.

These results indicate that the multiplication of Lee virus in the chick embryo

was markedly inhibited by the inoculation of non-infective Lee virus as long as 12 hours after the initiation of infection. A comparison of the virus titers found before and after the inoculation of non-infective virus suggests that the interference produced by the latter became effective within a very short interval after its introduction in the embryo.

*Effect of Inactivation of Influenza Viruses by Long Storage at 4°C. upon Their Capacity to Produce Interference.*—Two allantoic fluid pools, one containing Lee virus and the other containing PR8 virus, which had been stored at a temperature of 4°C. for 5 months, were tested for chick embryo infectivity. The Lee virus pool was found to be non-infective while the PR8 virus pool was found to be only partially inactivated. These preparations were tested for their capacity to produce interference.

Four groups of embryos were inoculated. Group 1 was given 0.1 cc. of undiluted partially inactivated PR8 virus, followed immediately by $10^2$ E.I.D. of active Lee virus. Group 2 was inoculated with 0.1 cc. of undiluted non-infective Lee virus followed immediately by $10^2$ E.I.D. of active PR8 virus. Group 3 was inoculated with $10^2$ E.I.D. of Lee virus, and group 4 with $10^2$ E.I.D. of PR8 virus. All of the embryos were incubated for 48 hours, and the allantoic fluids were then removed and tested in the usual manner.

The results were similar to those obtained with ultraviolet irradiated preparations. Of the allantoic fluids removed from embryos which had received PR8 virus partially inactivated by long storage at 4°C. followed by active Lee virus, none produced RBC agglutination. No RBC agglutination was produced by allantoic fluids from the embryos inoculated with Lee virus rendered non-infective by aging, followed by active PR8 virus. The allantoic fluids from the control embryos, which received either active PR8 or Lee virus alone, without exception produced RBC agglutination.

These results indicate that changes comparable to those induced in either Lee or PR8 virus allantoic fluid pools by exposure to ultraviolet radiation may also occur during storage for long periods at 4°C. Both procedures may result in the production either of partially inactivated or of non-infective influenza viruses which retain the capacity to produce interference in the chick embryo.

*Effect of Inactivation of Influenza Viruses by Heat upon Their Capacity to Produce Interference.*—Experiments were carried out to determine whether or not influenza viruses inactivated by heat were capable of producing interference with the multiplication of active virus in the chick embryo.

A number of allantoic fluid pools containing either Lee or PR8 virus were heated at 56°C. in a water bath. After various periods of heating, samples were removed from these pools and were tested both for the presence of active virus and for their capacity to produce interference in chick embryos. The methods used were identical with those employed in previous experiments with ultraviolet irradiated preparations.

The results obtained in these experiments require only brief discussion. In all instances it was found that when the period of heating had not been sufficient to inactivate the virus completely, interference could be demonstrated. On the other hand, when the period of heating was long enough so that active virus was not demonstrable, no unequivocal evidence was obtained that interference occurred. Under the conditions of these experiments it appeared, therefore, that heating at 56°C. destroyed the infectivity of influenza viruses as well as their capacity to produce interference in approximately equal time.

*Effect of Inoculation of Mice with Non-Infective Virus upon Subsequent Infection by Active Virus.*—The effects of influenza viruses, made non-infective by ultraviolet radiation, upon the multiplication of active virus in the chick embryo were sufficiently striking so that it seemed of obvious importance to determine whether the same phenomenon could be demonstrated in another species. In a number of experiments in which the intranasal inoculation of groups of mice with either non-infective PR8 virus or non-infective Lee virus was followed in 24 hours by the intranasal inoculation of the homologous active virus, evidence was obtained that the test group was resistant to no more than from 10 to 25 times the 50 per cent mortality dose of virus. It seems evident, therefore, that in these experiments the degree of interference produced by non-infective virus in the mouse was of a very different order of magnitude from that observed under similar conditions in the chick embryo.

## DISCUSSION

That ultraviolet radiation is capable of inactivating a number of different viruses without destroying their antigenic specificity is well known. The results obtained in this study indicate that influenza viruses rendered non-infective by ultraviolet radiation also retain the capacity of producing interference. Whether irradiated influenza viruses which appear to be non-infective, as evidenced by their failure to cause any indication of infection either in mice or on serial passage in chick embryos, are in fact wholly inactive and incapable of infecting susceptible tissues, seems impossible to determine with certainty. It may be that small amounts of virus in the preparations designated non-infective were still infective and actually capable of multiplication, even though the available evidence does not indicate that this was the case. However, the results obtained cannot logically be explained on the basis that they were dependent upon the presence of a small quantity of active virus in the non-infective preparations and experimentally they could not be reproduced by using virus at dilutions beyond the limits of detectability. In the preceding paper (1) it was shown that when small amounts of fully active influenza viruses were inoculated simultaneously no interference occurred. With non-infective preparations, however, not only did interference occur when these preparations were mixed with active virus and the two were inoculated together, but also

interference was demonstrable even when non-infective preparations were given after an active infection had been established. It was shown previously (1) that large amounts of active virus are required to produce a similar reversal in the direction of interference.

The failure to demonstrate effective interference between non-infective and active virus in the mouse makes it seem unlikely that significant amounts of active virus could be masked in this species by "auto-interference". The reinoculation with normal allantoic fluid by the intranasal route of mice previously given partially inactivated virus preparations by the same route accelerated the time of death of these animals, as was to be expected from the results reported by Taylor (7). However, these same partially inactivated preparations produced "auto-interference" on the first chick embryo passage. On the other hand, mice given non-infective virus preparations intranasally, and reinoculated with normal allantoic fluid, invariably survived and showed no pulmonary lesions.

Reciprocal interference between the strains of influenza A, influenza B, and swine influenza virus used in this study was as readily demonstrable with non-infective preparations as with the active viruses (1). However, no preparation of non-infective PR8 virus was obtained which produced effective interference with swine virus. Moreover, preparations of irradiated swine virus were not tested. In those instances in which it was determined it appeared that non-infective virus was as effective as active virus (1) in producing interference with the multiplication of medium sized inocula of active virus. The capacity of non-infective virus to interfere with large inocula of active virus was not measured, but it was found that the interference produced by partially inactivated virus could be overcome to some extent by large inocula of active virus. These findings correspond to those obtained with the active viruses (1). The only striking difference noted between the behavior of active and non-infective virus in the production of interference was that in the latter instance very large inocula were necessary to produce the desired results. This difference appears clearly to be related to the loss by non-infective virus of the property of multiplication.

In those respects in which they may be compared, there are close similarities between the interference produced by non-infective influenza viruses and the interference described by Luria and Delbrück (3) who used a strain of *E. coli* bacteriophage inactivated similarly by ultraviolet radiation. That interference was produced by ultraviolet irradiated, but only partially inactivated, influenza A virus has been reported by Henle and Henle (8). Jungeblut and Sanders (9) in discussing the effects of their murine virus upon the course of poliomyelitis in monkeys, stated that similar effects had been produced by murine virus which had been partially inactivated by ultraviolet radiation.

While it has been demonstrated that large amounts of non-infective influenza

viruses were necessary to produce interference, it has not been possible to define the quantitative relationships that probably exist between the actual amount of non-infective virus inoculated and the degree of inhibition of active virus multiplication produced.

The failure to arrive at a quantitative expression derives from the fact that it has not yet been possible to characterize non-infective virus preparations except in terms of the interference they produced. With exposure to ultraviolet radiation, certain stages in the process of virus inactivation could be demonstrated, but the time and the rate at which these occurred varied widely from one allantoic fluid pool to another. The observed variation in time of irradiation required to produce a certain stage of inactivation appeared to be unrelated to the initial virus titer, the RBC agglutination titer, the concentration of normal horse serum added, or the pH of the preparations. The cause of this variation has not been investigated, but it seems possible that it may be associated with the variations in the constitution of allantoic fluid which are known to occur (10).

The progressive stages reached during the process of inactivation by ultraviolet radiation were found to have the following characteristics with respect to the interference phenomenon. In the first stage the amount of active virus decreased markedly, but the interference produced was accompanied by multiplication of the residual active virus. When the proportion of non-infective virus to active virus was such that the multiplication of active virus was inhibited, the second stage of partial inactivation, the stage of "auto-interference" had been reached. The next demonstrable step was the apparent loss of the property of multiplication; no active virus could be demonstrated in mice or by serial chick embryo passage. In this non-infective stage the property of interference with either the homologous or heterologous viruses was retained. With still longer irradiation a fourth stage was reached in which an additional alteration was evident. At this stage both PR8 and Lee virus preparations were capable of interfering with the multiplication of Lee virus, but neither interfered significantly with the multiplication of PR8 virus. Finally, on prolonged irradiation a final stage in the alteration of virus was reached when interference could no longer be demonstrated.

The rate at which this series of changes occurred was much slower with Lee virus than with PR8 virus. It was not difficult to obtain specimens representative of each of the stages described with Lee virus. On the other hand, irradiation very rapidly caused alterations in PR8 virus. It was frequently found with specimens of PR8 virus from the same pool, that a period of irradiation, differing by only a few minutes from that insufficient to render the virus non-infective, caused the virus to be non-infective and also capable of causing interference. None of the PR8 virus preparations has shown "auto-interference", and only a few were both non-infective and capable of causing interference. Although

the stage of "auto-interference" was not demonstrated with PR8 virus, it seems not unlikely that the same sequence of changes occurs as with Lee virus, but at a rate so rapid that in order to obtain the virus at a desired stage of alteration it is necessary to sample the irradiated pool at the unknown proper moment.

The failure of exposure to heat to produce PR8 or Lee virus preparations which were non-infective but retained the capacity to produce interference, is similar to the experience of Luria and Delbrück (3) with bacteriophage interference. Henle and Henle (8), however, have reported recently that influenza virus incompletely inactivated by heat was capable of causing interference.

The demonstration that both non-infective virus and equally large amounts of active virus (1) caused interference when introduced simultaneously with active virus, and that non-infective virus was effective in inhibiting very rapidly the multiplication of active virus when it was inoculated after the establishment of infection, leads to certain suggestions regarding the mechanism of the interference phenomenon.

Since the conditions necessary for interference were produced with great rapidity after the introduction of non-infective virus or equally large amounts of active virus, the hypotheses which ascribe the interference phenomenon to pathologic changes within the cell, the exhaustion of essential metabolites by infection, or the production of antiviral substances, do not seem satisfactory for the system studied here.

The theory that infection by influenza virus is mediated by a cell receptor substance has been advanced by Hirst (11). It seems possible that such a receptor substance could be reactive with either active virus or with inactive virus if the latter retained the particular structural configuration required. Interference might then result from quantitative saturation of the receptor substance of susceptible cells, whether the point of saturation was achieved by the multiplication of small amounts of active virus or by the introduction of large amounts of virus, either active or non-infective.

The demonstration of interference between antigenically distinct viruses, notably between those of Rift Valley fever and yellow fever, as well as between the viruses of lymphocytic choriomeningitis and poliomyelitis, virus III and the Shope fibroma virus, and the recently observed example of interference between Theiler's virus and Western equine encephalomyelitis virus (12), suggests further considerations. It has been considered probable, particularly in the case of plant viruses, that interference between two viruses is evidence of biological relationship. Delbrück and Luria (2) have suggested that reciprocal interference proves relatedness, and that with unrelated viruses interference will be effected by only one of the viruses, or by neither. However, in a number of instances there is evidence that reciprocal interference occurs between very different viruses. Indeed, the failure to demonstrate reciprocal interference

may depend on such factors as time, the relative virulence of the two viruses for the test species, or the unavailability of methods for the detection of interference except in a single direction. Virus combinations in which interference is demonstrable may consist of agents which also appear by other criteria to be related. Moreover, in all known instances of interference both viruses share at least a common tissue tropism, although evidence may be lacking that they actually infect identical cells. Despite these facts, it seems unwise to assume that reciprocal interference necessarily indicates biological relationship, since it may indicate merely that the viruses concerned are each capable of reacting with a single theoretical receptor substance possessed by cells of the susceptible tissue. On this basis it can be anticipated that closely related although antigenically distinct viruses may show mutual interference, as is the case with the influenza viruses. On the other hand, it seems entirely possible that reciprocal interference may be demonstrable between viruses which by all other available criteria appear to be unrelated.

A similar antigenic constitution is evidently not the basis of interference between the influenza viruses, and the capacity of these agents to produce interference seems not to be essentially a function of the property of multiplication. Consequently, it appears reasonable to assume that reciprocal interference between the influenza viruses depends upon some portion of the virus particles capable of reacting with susceptible cells, possessed in common by influenza A, influenza B, and swine influenza viruses and relatively resistant to ultraviolet radiation. Regarding the nature of this portion of the virus particle, there is as yet no evidence.

## CONCLUSION

Influenza A or influenza B virus rendered non-infective by ultraviolet radiation was found to be capable of producing interference with the multiplication of active influenza viruses in the chick embryo. Certain temporal and quantitative relationships affecting the interference phenomenon with this host-virus system were studied. An hypothesis of the mechanism of interference between the influenza viruses is proposed and discussed.

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