FURTHER STUDIES ON INTERFERON

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IN a series of studies of the viral interference reaction it was found that a product of the interference phenomenon, called interferon, was formed and was itself capable of inducing interference (Isaacs and Lindenmann, 1957). Interferon appears to be a protein which is taken up by chorioallantoic membranes rendering them unable to support viral multiplication (Lindenmann, Burke and Isaacs, 1957). In this paper we report further attempts to characterize interferon and to describe the conditions for its production.

MATERIALS AND METHODS

These were generally those of the previous paper (Lindenmann *et al.*, 1957) except that ultra-violet irradiated (u.v.) virus was used routinely for the preparation of interferon. The virus strains used were the classical Melbourne and PR8 strains of influenza A, the influenza strain A/Persia/2/52 which produces abundant filaments, and the haemagglutinating virus of Japan or Sendai virus (Kuroya, Ishida and Shiratori, 1953). The latter strain was used whenever only small amounts of interferon were present because of its sensitivity to interferon. Incomplete influenza virus was prepared by the technique of von Magnus (1951) in which three passages of undiluted infective allantoic fluid were made in the allantoic cavity. Third passage virus was found to have a ratio of infectivity/haemagglutinin titre of $< 0{\cdot}1$ per cent of standard virus.

Enzymes: pepsin, a crystalline preparation of porcine origin from Armour & Co. (lot 12655) and ribonuclease, a crystalline preparation from Worthington Biochemical Co. (lot A546). Phosphorus was determined by a micro modification of the method of Allen (1940).

RESULTS

Dosage of heat and u.v.-inactivated virus.

It has been previously observed (Lindenmann *et al.*, 1957) that u.v.-inactivated virus produces more interferon than heat-inactivated virus and an experiment was designed to examine quantitatively their relative efficiency in producing interferon and the effect of dosage with the two types of virus used. Heat and u.v.-inactivated virus were adjusted to the same haemagglutinin titre (1200) and the different doses, made up to 50 ml. with buffer, shaken for 3 hr. at 37° with 5 whole chorioallantoic membranes in 1 litre Roux bottles. The membranes were washed, fresh buffer was introduced and they were then shaken overnight to produce interferon in the usual way. Titration of the fluids gave the results shown in Fig. 1 in which the dose of virus used is plotted against the yield of interferon produced.

It can be seen that u.v.-inactivated virus is much more efficient in producing interferon than an equivalent amount of heat-inactivated virus. Since it was not clear whether maximal production of interferon had been reached at a dose of

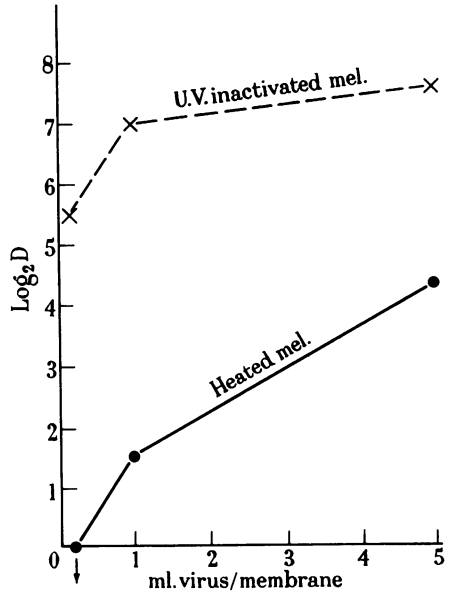

FIG. 1.—Production of interferon by varying doses of heat and u.v.-inactivated virus.

5 ml. of heated virus per membrane, the amount of interferon produced by a wider range of heat-inactivated virus was determined. The results are shown in Table I.

TABLE I.—*Production of Interferon by Heat-inactivated Influenza Virus*

Group No.	Material tested	Virus yield after challenge: $-\log_2$ HA titre*	Virus yield after challenge: Per cent of control group
1	1 ml. heated MEL/membrane	6·4	50
2	2 ml. heated MEL/membrane	3·0	4·7
3	4 ml. heated MEL/membrane	2·5	3·3
4	8 ml. heated MEL/membrane	1·1	1·4
5	Buffer	7·4	100

* Expressed as geometric mean titre of dilution end-point ($-\log_2$) in this and in the following tables.

This suggests that heat-inactivated virus is capable of producing as much interferon as u.v.-inactivated virus, but at a much higher dosage per membrane.

Attempted preparation of interferon directly from inactivated virus

An attempt was made to determine whether interferon was a part of the infecting virus or not, by sonic disruption of influenza virus filaments and examination of the fluid for interfering activity. A/Persia/2/52 allantoic fluid was centrifuged at 1000 *g* for 30 min. and the deposit suspended in one-tenth the volume of saline in order to concentrate filaments. The fluid was divided into three portions; one was irradiated with u.v. light for 2 min. under the usual conditions, the second

was irradiated as before and followed by ultra-sonic treatment (six half-minute bursts of 350 k.c.), while the third was given ultra-sonic treatment only. The latter two portions were dialysed with stirring against an 0·05 M pH 1 buffer for 2 hours to destroy intact virus and then dialysed against pH 7·4 buffer overnight. Interferon is known to be stable on treatment at pH 1 in this way.

The fluids were then assayed for their interfering activity (in the usual way except that Sendai virus was used for challenge) with the result shown in Table II.

TABLE II.—*Attempted Preparation of Interferon from Inactivated Virus*

		Material tested	Virus yield after challenge	
			— $\log_2$ HA titre	Per cent of control group
Group No.	1	u.v.-irradiated	1	12
	2	„ „ , sonics and pH 1	5·3	158
	3	Sonics and pH 1	4·3	113
	4	Buffer control	4·1	100

If any interferon had been liberated by treatment with ultra-sonics it would have been stable at pH 1 (Lindenmann *et al.*, 1957) and should have been detected in group No. 2. There is thus no evidence from this experiment to suggest that interferon is part of the inactivated virus.

Production of interferon from live and u.v.-irradiated virus on the Chorion

Heat-inactivated virus has been shown to produce interferon when inoculated on to the chorionic surface (Lindenmann *et al.*, 1957) and it was of interest to see whether live virus would produce interferon in this system. The first experiments showed that barely significant amounts of interferon were being produced and so the following experiment was set up. Freshly harvested PR8 allantoic fluid was adsorbed on to 1 per cent red cells and eluted into half the volume of saline. The eluate (haemagglutinin titre 2400–4800) was irradiated with u.v. light in 3 ml. portions for 0·5, 1, 2, 4 and 8 min. and infectivities were determined on these fluids and on the unirradiated eluate. Groups of 11-day eggs were inoculated on the dropped chorionic surface with 0·5 ml. of each of the irradiated fluids and of unirradiated eluate (4 eggs per group). After incubation at 37° for 90 min. 0·1 ml. of PR8 serum (heated at 56° for 30 min.) was added to each egg. After a further 30 min. incubation the eggs were opened, the chorions removed and washed, and the pooled membranes from each group incubated at 37° on the roller drum overnight with 2 ml. of buffer and 0·1 ml. of antiserum. Under these conditions no haemagglutinin was produced. The fluids were diluted with buffer to 6 ml. and tested for the presence of interferon using Sendai as challenge virus. Serological tests showed that only Sendai virus was produced. The results of two experiments are shown in Fig. 2 as a plot of time of irradiation against interferon yield ; in the first experiment the irradiated materials had no infectivity while in the second, some of the fluids showed a very low infectivity ($< 10^{-1}$) and there was no clear relationship apparent between loss of infectivity and production of interferon.

These results show that short periods of irradiation led to maximal production of interferon but that on further irradiation, or with unirradiated material, very

little interferon was produced. Two other experiments gave essentially similar results.

Production of interferon by complete and incomplete virus on the chorion

Since interferon was only produced after loss of infectivity it was decided to compare the efficiency of complete and incomplete virus in producing interferon on the chorion. It is known that incomplete virus is capable of inducing interference provided sufficient time is allowed before challenge (von Magnus, 1954).

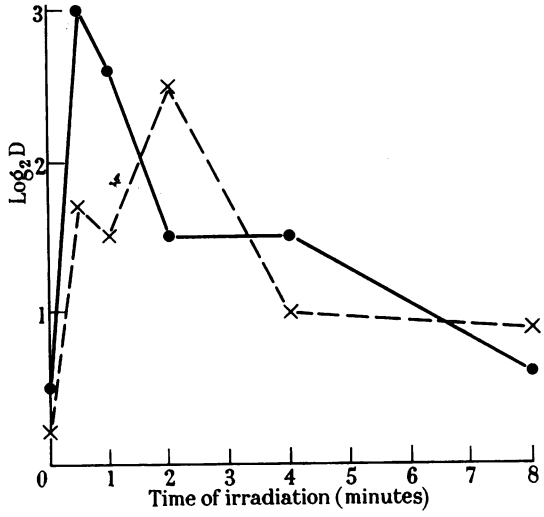

FIG. 2.—Production of interferon on the chick chorion by virus irradiated with u.v. for varying times. First experiment ———, second experiment .....

Accordingly, incomplete (third undiluted passage) and complete (diluted passage) PR8 virus were adjusted to the same haemagglutinin titre and 1 ml. portions inoculated on to the dropped chorion of 11-day eggs (4 per group). The eggs were treated as described in the previous experiment with the results shown in Table III.

TABLE III.—*Production of Interferon by Complete and Incomplete Virus on the Chick Chorion*

Materials tested	I.D.$_{50}$/0·05 ml.	Virus yield after challenge: $-\log_2$ HA titre	Virus yield after challenge: Per cent of control
Complete PR8	$10^{9.5}$	2·3	47
Incomplete PR8	$10^{5.8}$	0·1	11
Buffer control	..	3·4	100

Another experiment gave an essentially similar result.

Attempted preparation of an interferon competitor

One explanation of the failure of live virus to produce interferon when inoculated on to the chorion was that damaged virus produced interferon which was

a competitor with a normal component of the multiplication cycle of live virus. To test this, 1 ml. portions of infective PR8 virus were inoculated on to the dropped chorion of 11-day eggs and then treated as in the last two experiments. Both the fluids and a membrane extract (eight membranes ground with glass powder and 5 ml. of buffer followed by light centrifugation) were tested for the presence of a possible interferon competitor by mixing with a standard interferon preparation and testing for interfering activity in the usual way. No inhibitor of interferon activity was found in such membrane fluids or extracts. However, we cannot rule out the possibility that an inhibitor of interferon activity is produced in the membrane and is rapidly used up.

Miscellaneous

Confirmation of the protein nature of interferon was obtained by digestion with pepsin. Interferon was adjusted to pH 2 by dialysis against a 0·05 M potassium chloride/hydrochloric acid buffer and half was digested with crystalline pepsin (final concentration 0·001 per cent) at 37° for one hour, while the remainder was incubated at 37° as a control. This was followed by dialysis against pH 7·4 buffer and testing for interferon with the results shown in Table IV.

TABLE IV.—*Action of Pepsin on Interferon*

		Material tested	Virus yield after challenge: $-\log_2$ HA titre	Virus yield after challenge: Per cent of control
Group No.	1	Interferon	2·1	0·9
	2	Buffer + Pepsin	8·9	100
	3	Interferon + Pepsin	9·1	113

Interferon was not affected by prolonged digestion with ribonuclease (in saline at a final concentation of 0·001 per cent for 24 hr. at 37°) or by addition of sodium iodoacetate (at a final concentration of 0·01 M for 30 min. at room temperature), but it was completely destroyed by shaking for 5 min. with an equal volume of amyl alcohol/chloroform (1 : 2). It was only slowly inactivated by irradiation with u.v. light, (maximal emission at 2537 Å) as shown in Table V.

TABLE V.—*Effect of Ultra-Violet Irradiation on Interferon*

		Material tested	Virus yield after challenge: $-\log_2$ HA titre	Virus yield after challenge: Per cent of control
Group No.	1	Interferon	1·3	1
	2	,, irradiated 5 min.	2·2	1·8
	3	,, ,, 10 ,,	2·9	2·9
	4	,, ,, 20 ,,	3·8	5·4
	5	Buffer	8·0	100

This behaviour is not inconsistent with the formulation of interferon as protein or partially protein.

In a previous paper (Isaacs, Lindenmann and Valentine, 1957) the heat lability of interferon was advanced as an argument against its being a ribonuclease. Mr. N. W. Pirie has pointed out to us that not all ribonucleases are heat stable,

and to settle this point a direct assay of an interferon preparation for ribonuclease activity by a standard procedure was carried out (Colowick and Kaplan, 1955). A typical interferon preparation contained less than the equivalent of 0·15 μg. of pancreatic ribonuclease per ml. and this amount is insufficient to induce significant interference in the system used. Interferon would have to be at least thirteen hundred times more active than pancreatic ribonuclease on a weight basis to account for the degree of interference found.

DISCUSSION

From previous work it has not been possible to decide whether interferon is part of the inactivated virus used in its preparation, or whether it is newly synthesized in the membrane. The time of appearance of interferon and the presence of an eclipse phase (Isaacs and Lindenmann, 1957) favoured the second interpretation and this is supported by the effect of different dosages of heat and u.v.-inactivated virus on the yield of interferon. The flattening of the curve obtained with u.v.-inactivated virus (Fig. 1) could be due either to the inability of the cells to handle more than a limited number of viral particles or to a limited ability to synthesize interferon. However, the results with heat-inactivated virus make it clear that the number of viral particles is not the limiting factor. This suggests that interferon is newly synthesized in the membrane, but a firm decision on this point may require the use of radioactive labelled virus and membranes. There is some evidence that the saturation effect is a temporary one. We have already found that membranes given a second inoculum of inactivated virus produced a second crop of interferon (Lindenmann *et al.*, 1957) and more recently we found that third and fourth crops of equal potency can be obtained in the same way.

The results obtained with u.v.-irradiated virus on the chorion suggest that the virus particles must be slightly damaged before interferon can be produced. It is known (Powell and Setlow, 1956) that the rapid loss of viral infectivity on u.v.-irradiation is primarily due to damage of the viral nucleic acid and although there does not seem to be any relationship between loss of infectivity and interferon production, the short times of irradiation at 2537 Å necessary to obtain maximal yields of interferon suggest that the damage discussed above is in the nucleic acid or nucleoprotein of the virus. This idea is supported by the results obtained with incomplete virus since the most significant difference between incomplete and complete virus is the lower nucleic acid content of incomplete virus (Ada and Perry, 1956). The fact that incomplete virus has been found to be a much more efficient producer of interferon than complete virus again suggests that it is viral particles with damaged nucleic acid or nucleoprotein that initiate interferon production. However, excessive damage with u.v.-irradiation greatly reduced the amount of interferon produced. This suggests that interferon is an analogue of some intermediate normally synthesized during the growth of virus. If there is such an intermediate, we have until now been unable to demonstrate it.

SUMMARY

The production of interferon by varying amounts of heat and u.v.-inactivated virus has been examined. The results suggest that interferon is newly synthesized in the membrane, rather than part of the inactivated virus.

Interferon was produced by u.v.-inactivated virus on the chick chorion. Live influenza virus grown under similar conditions or heavily irradiated virus did not produce interferon, whereas incomplete virus produced good yields. The findings suggest that interferon is produced by virus in which the nucleic acid has been slightly, but not heavily, damaged.

We should like to thank Dr. C. H. Andrewes, F.R.S., for his advice and criticism and Mr. V. G. Law and Miss V. Coker for their technical help.

Recently Dr. F. Fulton pointed out to us that in estimating the size of interferon by filtration (Isaacs, Lindenmann and Valentine, 1957) we had not diluted interferon in broth. We therefore filtered some interferon, diluted in buffer, through a 0·5μ A.P.D. membrane and then filtered some of this material diluted in broth through the same membrane. Interferon activity was absent from the first filtrate but present in the second. In subsequent experiments we found that when interferon was diluted in broth and filtered through a collodion membrane of A.P.D. 0·048μ some interferon activity passed through the membrane. Thus the earlier filtration measurements were in error and this subject is being further investigated.

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