INTERFERON

Ciba Foundation Symposium
Dedicated to Alick Isaacs, F.R.S.

Edited by
G. E. W. WOLSTENHOLME
and
MAEVE O'CONNOR

J. & A. CHURCHILL LTD.
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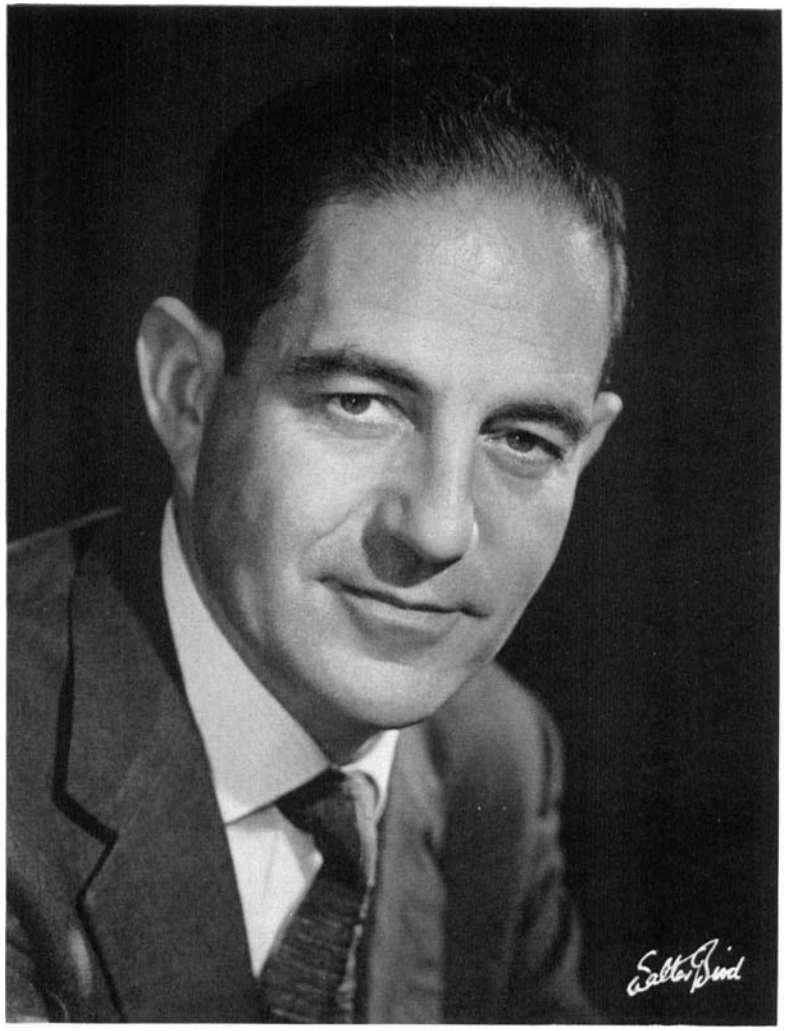
1968

INTERFERON



[Photograph by Walter Bird

ALICK ISAACS, 1921–1967

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The Ciba Foundation

The Ciba Foundation was opened in 1949 to promote international co-operation in medical and chemical research among scientists from all parts of the world. Its house at 41 Portland Place, London, has become a meeting place well known to workers in many fields of science. Every year the Foundation organizes from six to ten three-day symposia and three or four one-day study groups, all of which are published in book form. Many other informal meetings are held in the house, organized either by the Foundation or by other scientific groups needing a place to meet. In addition, bedrooms are available for scientists visiting London, whether or not they are attending a meeting in the building.

The Ciba Foundation owes its existence to the generosity of CIBA Ltd, Basle, who, realising the disruption of scientific communication caused by the war and by problems of distance, decided to set up a philanthropic institution whose aim would be to overcome such barriers. London was chosen as its site for reasons dictated by the special advantages of English charitable trust law (ensuring the independence of its actions), as well as those of language and geography.

The Foundation's many activities are controlled by a small group of distinguished trustees. Within the general framework of biological science, interpreted in its broadest sense, these activities are well summed up by the Ciba Foundation's motto, *Consocient Gentes*—let the nations come together.

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Preface

In his opening remarks here, Michael Stoker speaks of the remarkable way in which Alick Isaacs, in his short and brilliant life, was responsible for so much of the research and inspiration which formed the background to this meeting. It had earlier been a disappointment to us at the Ciba Foundation when Alick Isaacs had been too ill to take part in a conference on Myxoviruses in 1964; when his health improved and he suggested a symposium on Interferon, we seized the chance of marking the tenth anniversary of its discovery, when we could pay tribute to work of such importance done almost on our doorstep, at a time when the development of interferon seemed full of fresh promise.

Alick joined enthusiastically in the detailed organization of the symposium, but sadly it soon became obvious that he would not be fit to play a full leading role in it, and his death came just when the preparations were complete.

This symposium is therefore a gesture of thankfulness to Alick Isaacs both for his work and his friendship. The tribute to him will be all the more meaningful if these papers and discussions awaken in even one reader ideas for further development in this field of research.

We are most grateful to all who have contributed to this occasion, in particular to Michael Stoker for his appropriately friendly chairmanship, and to Joseph Sonnabend for his generous and expert help in editing the discussions.

CHAIRMAN'S OPENING REMARKS

M. G. P. STOKER

We are here to discuss interferon, and the one person who is missing is the person who is most responsible for bringing us together. We would not be here if Dr. Alick Isaacs and Dr. Lindenmann had not discovered interferon. We would not be here if Dr. Isaacs, with Dr. Wolstenholme and the Executive Council of the Ciba Foundation, had not initiated this particular symposium. Alick Isaacs died early this year, and the shock was particularly great to those of us who are here. It is appropriate, and certainly our wish, that the symposium and the book should be dedicated to him.

Dr. Isaacs' career and contribution to science have been, and are still being, reviewed by others much more competent than I am. In particular, Sir Christopher Andrewes is preparing an authoritative obituary which will be published by the Royal Society. I am certainly not going to review the history of interferon in detail before a company of acknowledged experts in this field—and I include amongst the acknowledged experts Dr. Sue Isaacs. Although pre-eminent in her own field, she must know more about interferon than a great many virologists. We are very happy that she is with us on this occasion at the beginning of the meeting, and that she will also be with us on some subsequent occasions during the next few days.

Even if interferon had never been discovered, Dr. Isaacs would still be remembered as a very eminent virologist: his work, which he continued for over twenty years with the utmost vigour, gave him a very considerable reputation. This particularly applies to his studies on influenza virus in all its aspects, from epidemiology to virus structure. But the supreme event in his scientific life was the publication of two papers, one with Dr. J. Lindenmann (1957. *Proc. R. Soc. B*, **147**, 258) and the other with Dr. Lindenmann and Dr. R. C. Valentine (1957. *Proc. R. Soc. B*, **147**, 268). It falls to very few of us to make a single outstanding contribution which has such a tremendous repercussion over many years and which leads to innumerable meetings, books, and not least to a Ciba Foundation symposium ten years later.

I first heard about interferon in a pub near University College which I am sure is familiar to many of us—it ought to be called "The Grapevine":

Dr. Isaacs and I went there as usual after a meeting; he was obviously excited and out came the idea of interferon. A lot of very interesting scientific gossip may be heard in that particular pub, but I suppose this was the most important thing I ever heard there.

After the original discovery and publication, he threw himself with typical and enormous energy into both the practical and theoretical aspects of the subject, and did indeed lay much of the ground work for what was to follow. The idea of interferon immediately had great appeal, not only amongst medical people, who saw it as a possible therapeutic weapon, but also amongst virologists who were concerned with basic aspects of virus-cell interaction; despite this appeal, however, it was not taken up experimentally in many other laboratories outside Mill Hill for several years. In fact there were centres where the whole idea was criticized and regarded rather cynically. Many of us will remember the word "misinterpreton" which was circulating. It is true, of course, that some of Dr. Isaacs' theories and ideas have subsequently turned out to be wrong, as happens to all of us. We know now that some of his observations were due to impurities in early preparations, and now it is much easier to interpret things. But the basic idea was not wrong: it was not "misinterpreton". He had enormous faith in interferon, and his enthusiasm and faith kept it going in the two or three, perhaps rather lonely, years when it was being criticized and not taken up very widely. Eventually, of course others entered the game with equal enthusiasm. This has led in the last few years to the second phase of interest in interferon, with tentative interpretation of its production and action in the context of modern molecular biology. If Alick Isaacs had not kept the project going during the early period, however, there might have been a very long delay before this second expansion of investigation took place, and I feel that he should be remembered for this as well as the original observation.

You will perhaps wonder why I am here as chairman. There are two kinds of chairman. One is the authoritative figure who knows the contents of every paper in advance, who knows who is going to ask what question in the discussion and what the answer is going to be; this is the paternalistic type of chairman. I am the other sort: indeed I must be one of the few virologists who has never published a paper on interferon. In fact I did once do some work on interferon, and was about to post an article to a journal, but on re-reading it I decided that the subject was much to difficult and complicated. So I tore it up—it is the only paper I have destroyed at that stage.

Dr. Isaacs is responsible for my being chairman, and I suspect he knew in his kindly way that amongst his colleagues and friends, I was the one who

most needed educating. He was absolutely right, and I am going to enjoy being educated by you all during the coming three days.

Finally, may I remind you of Alick Isaacs' main characteristic—his joyful and bubbling enthusiasm, described very movingly by Dr. John Humphrey at the memorial ceremony. If this meeting is to be dedicated to Alick Isaacs it would be no service to him if we made it a funereal and mournful occasion. On the contrary we should proceed with the light-hearted enthusiasm which he himself would have shown.

CELLULAR EVENTS PRECEDING INTERFERON FORMATION

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VIRUS-INDUCED interferon formation may be represented as follows (Fig. 1). The virus, which may be either infective or inactivated, invades

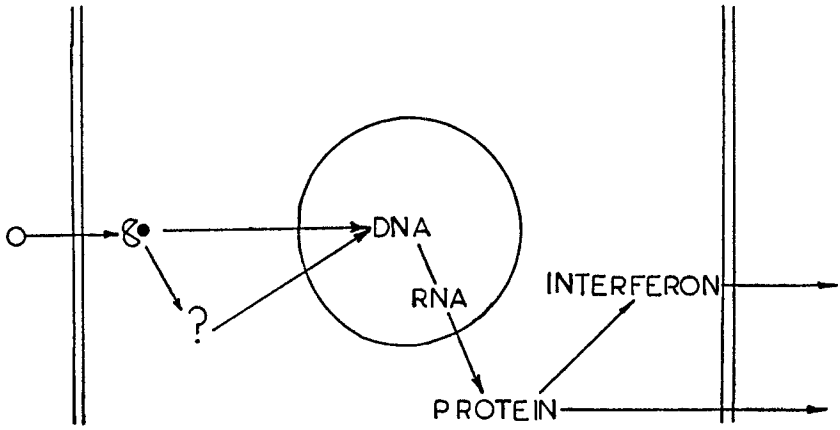

FIG. 1. A hypothetical scheme for interferon production.

the cell and presumably uncoats, although we do not know whether uncoating is necessary or not. Then some virus component, or some product of the virus-cell interaction, proceeds to the nucleus and there initiates, by a process similar to derepression, the formation of an interferon messenger RNA. This RNA then codes for the protein interferon, or possibly for a precursor of interferon. This scheme is no more than a working hypothesis, but it does accommodate all the available evidence, and provides a framework for the interpretation of results.

THE NATURE OF THE INTERFERON INDUCER

What component of the virus is responsible for the induction of interferon formation—the virus protein, virus nucleic acid, or some other component or combination of components? The inducer is more stable to

heat and ultraviolet irradiation than virus infectivity, since non-infective viruses which can still induce interferon formation can readily be prepared. However the ability of several myxoviruses to produce interferon is destroyed by increased ultraviolet irradiation; therefore it is likely that the inducer absorbs ultraviolet light, and is probably protein or nucleic acid. The production of low titres of inhibitory material by treatment of cells with nucleic acids led Isaacs (1963) to the concept of "foreign nucleic acid" as the inducer for interferon formation. However the inhibitory material was never fully characterized, and other workers have found it difficult to repeat these observations. It is possible that this inhibitory material is similar to the preformed material released after treatment of animals with bacterial endotoxin.

We have made two attempts to identify the nature of the interferon inducer. The first approach was to see whether isolated virus nucleic acid

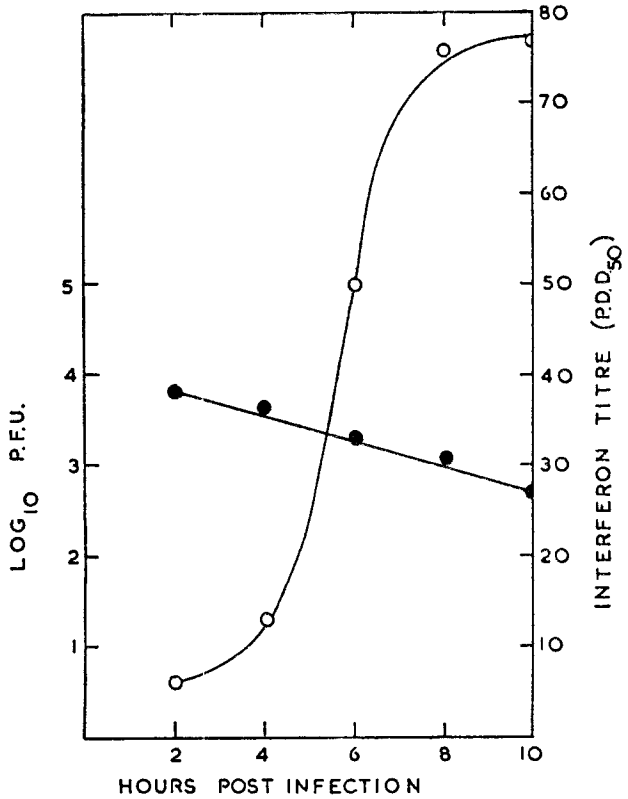

FIG. 2. The production of virus (—●—●—) and interferon (—○—○—) when chick cells were incubated at 42° after infection with SFV for 1 hr. at 37°.

would induce interferon formation. In order to obtain an unequivocal answer it was necessary to use a biological system in which interferon but not virus protein was formed. Infection of chick embryo cells with Semliki Forest virus (SFV) at 37° C, followed by incubation at 42°, was satisfactory for this purpose, since at 42° interferon but not virus was formed (Fig. 2), and we have been unable to detect any virus-induced protein synthesis at 42° (see below). SFV readily yielded an infective nucleic acid (Cheng, 1958) and it was thus possible to obtain an undegraded nucleic acid from a virus capable of inducing interferon formation. However a high virus multiplicity was necessary in order to obtain maximum interferon yields (Table I), and this meant that very large amounts of

TABLE I

EFFECT OF VIRUS MULTIPLICITY ON VIRUS AND INTERFERON YIELD

Multiplicity of exposure (*p.f.u. SFV/cell*)	*Virus yield per culture* (log_{10}/*p.f.u.*)	*Interferon yield per culture* (PDD_{50})
$3{\cdot}6 \times 10^2$	8·88	84
$1{\cdot}2 \times 10^2$	8·55	98
$4{\cdot}0 \times 10^1$	8·66	40
$1{\cdot}3 \times 10^1$	8·38	30
4·5	8·50	12
1·5	8·50	4

Chick cells (7×10^6) were infected with varying multiplicities of partially purified SFV for 1 hour at 37°. Virus was harvested after 8 hours at 37°, and interferon after 12 hours at 42°.
p.f.u.: plaque-forming units.
PDD_{50}: plaque-depressing doses.

infective virus nucleic acid would have to be prepared. In addition, the high molarity salt treatment, needed to force virus nucleic acid into the cells, depressed the virus-induced interferon response, and it was decided to attempt another approach.

Hydroxylamine has been shown to inactivate the infectivity of a number of viruses through reaction with the virus nucleic acid, without any effect on viral antigenicity (Schafer and Rott, 1962). Recently Grossgebauer (1966) has found that hydroxylamine inactivated both the infectivity and interfering ability of influenza virus without any effect on antigenicity. We have examined the effect of hydroxylamine inactivation of SFV on its interferon-inducing capacity in order to obtain more information about the nature of the inducer. The inactivation of partially purified SFV by 0·2 M-hydroxylamine at pH 7 and room temperature was a first-order reaction, in a typical experiment, the virus titre falling four log_{10} units in five hours (Fig. 3). Samples of virus were removed after inactivation for varying times, and after dialysis they were examined for their ability to

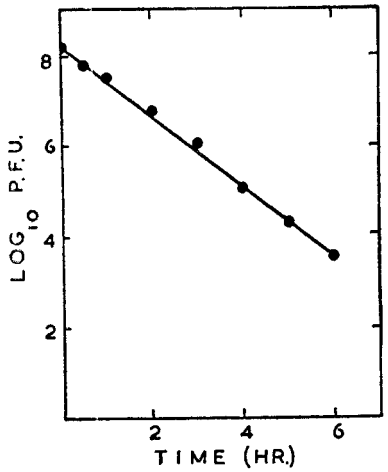

FIG. 3. Inactivation of SFV by 0·2M-hydroxylamine.

produce infectious virus, virus haemagglutinin, virus-induced RNA synthesis (Taylor, 1965) and virus-induced RNA polymerase (Martin and Sonnabend, 1967). There was no effect of hydroxylamine inactivation until the multiplicity of infection fell below one, when all these virus-induced properties were inactivated with first-order kinetics (Fig. 4).

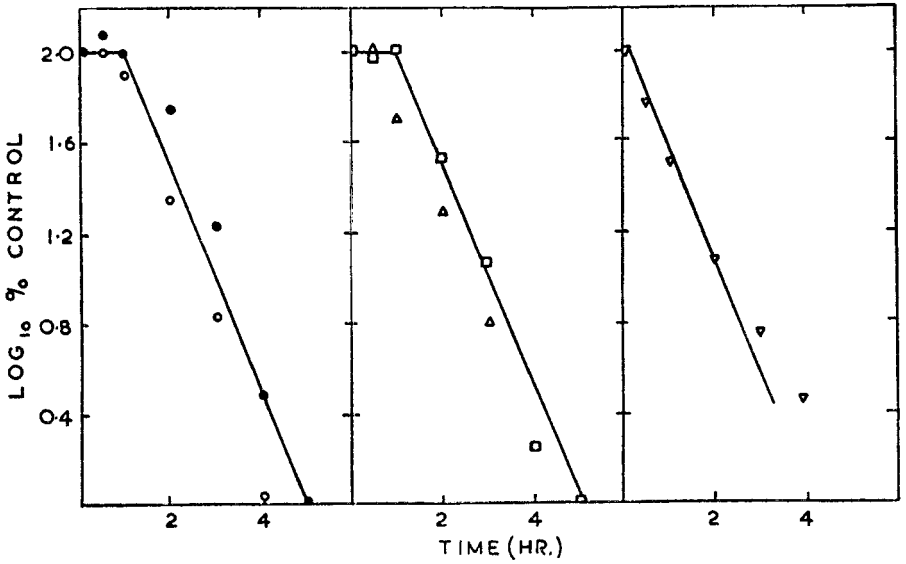

FIG. 4. The production of infectious virus (—●—●—), virus haemagglutinin (—○—○—), virus-induced RNA synthesis (—□—□—), virus-induced RNA polymerase (—△—△—), and interferon (—▽—▽—) by SFV inactivated for different times with 0·2M-hydroxylamine.

The inactivated virus samples were also examined for their interferon-inducing capacity by incubation of cells at 42° after infection at 37°. The interferon-producing capacity was inactivated by hydroxylamine at the same rate as that of the other virus-induced properties, suggesting that complete virus nucleic acid is essential for induction of interferon production by this virus.

EARLY STAGES IN INTERFERON FORMATION

In a number of systems virus multiplication and interferon production proceed concomitantly, although either one may be selectively inhibited in various ways. If both processes depend on intact virus nucleic acid for initiation, how much do the two processes have in common? Are some of the early stages of virus multiplication also necessary for interferon formation, and if so, which stages? We have looked for evidence of involvement of the early stages of virus multiplication in interferon production by SFV at 42°.

Viral nucleic acid synthesis is readily detected by treating the cells with actinomycin and pulsing with radioactive uridine (Taylor, 1965), and we have used this method to look for viral nucleic acid synthesis early in interferon production. The treatment with actinomycin inhibited subsequent interferon formation, but it was assumed that any necessary viral nucleic acid synthesis occurred before the actinomycin-sensitive step

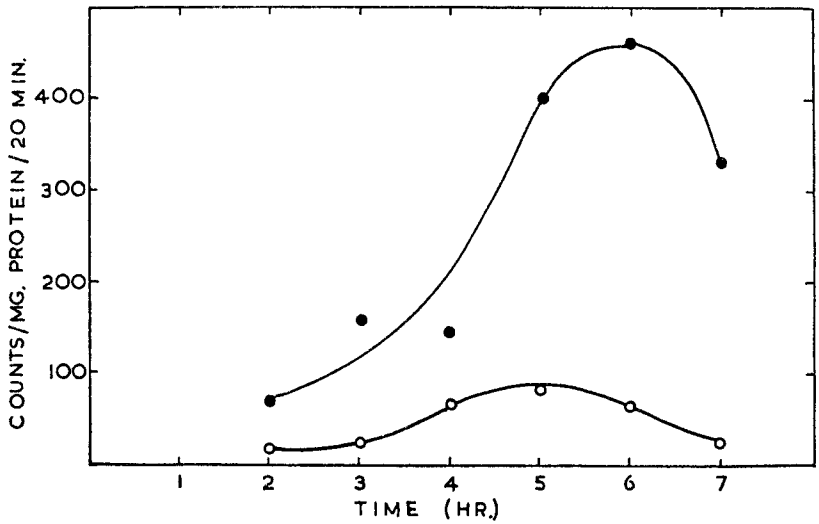

FIG. 5. Production of virus-induced RNA polymerase when chick cells were incubated at 37° (—●—●—) or 42° (—○—○—) after infection with SFV for 1 hr. at 37°. The enzyme was assayed at 37° essentially as described by Martin and Sonnabend (1967).

in interferon production. No viral nucleic acid synthesis could be detected and it was concluded that viral nucleic acid synthesis was unnecessary for interferon production (Burke, Skehel and Low, 1967).

A stage prior to viral nucleic acid formation is the formation of the virus-induced RNA polymerase. This protein appears to be the first virus-coded property to be expressed. We therefore looked for virus-induced RNA polymerase in SFV-infected cells at 37° and at 42°. Fig 5 shows that polymerase was detectable when the cells were incubated at 37° but not at 42°, and therefore this polymerase is not necessary for interferon formation. The failure to find polymerase at 42° could be due to inhibition of either its formation or its action by incubation at the increased temperature, since polymerase formation is autocatalytic. However, the polymerase formed at 37° was active when assayed at 42°, suggesting that the enzyme is not *formed* at 42°. The product formed by this enzyme is predominantly double-stranded RNA (Martin and Sonnabend, 1967), and if two polymerases are involved in virus RNA replication, it is probably the first to be formed. Thus the pathways that lead to virus multiplication and interferon production part very early after infection—at least before formation of the polymerase.

It was possible that other virus-coded proteins were formed as an early stage of interferon formation, and a search was made for these, using radioisotopes. No stimulation of the rate of protein synthesis could be detected after infection of chick cells with SFV at 37° and only a small stimulation was detectable when actinomycin-treated cells were used (the inhibitor was added to depress host-cell protein synthesis). This was presumably because viral protein synthesis represented only a small portion of the total protein synthesis. However the synthesis of virus-induced proteins could be detected by fractionation of the proteins by polyacrylamide gel electrophoresis (Summers, Maizel and Darnell, 1965). SFV-infected cells were pulse-labelled with [^{3}H]valine and control cells with [^{14}C]valine at intervals after infection. The harvested cells were mixed and fractionated by polyacrylamide gel electrophoresis. Measurement of the [^{3}H]/[^{14}C] ratio revealed the presence of several virus-coded proteins, two of which have been identified as components of the virus coat protein (Fig. 6). In this system, mobility is governed largely by molecular size, and the fast-moving components around fraction 60 are probably incomplete polypeptide chains. The other components have not yet been identified. When cells were incubated at 42° after infection, none of these proteins could be detected and we concluded that the formation of virus-coded proteins was not essential for interferon production.

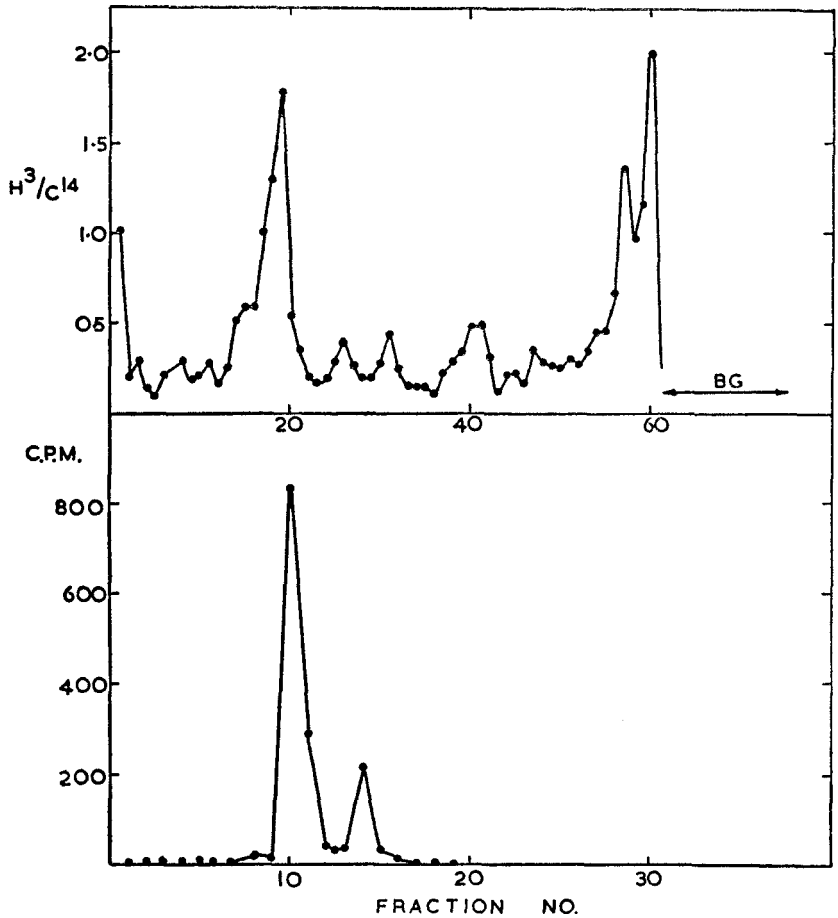

Fig. 6. (*a*) Production of virus-induced proteins in SFV-infected cells. Chick cells were treated for 4 hr. at 37° with 0·5 μg./ml. of actinomycin, infected with SFV for 1 hr. at 37°, and then incubated at 37°. Isotope was added at hourly intervals for 8 hr.; the figure shows the result obtained when it was present from 6 to 7 hr. after infection (BG=background counting rate).

(*b*) Proteins from SFV. The virus was grown in actinomycin-treated cells in the presence of [^{3}H]valine, purified as described by Cheng (1961), and the proteins extracted and fractionated as in (*a*).

SYNTHESIS OF THE INTERFERON MESSENGER RNA

The inhibition of interferon production by actinomycin (Heller, 1963) suggests that the formation of the messenger RNA for interferon synthesis is directed by cellular DNA rather than by the virus nucleic acid. This interpretation accounts for the frequent observations of the species specificity of interferon (Lockart, 1966), and the induction of an apparently identical interferon by either a DNA or an RNA virus (Lampson *et al.*,

1965), but it assumes that actinomycin inhibits interferon production by acting solely as an inhibitor of DNA-dependent RNA synthesis. Actinomycin, however, exerts a number of other effects, including inhibition of DNA synthesis (Baserga *et al.*, 1965), inhibition of RNA transfer from the nucleus to the cytoplasm (Levy, 1963), and inhibition of protein synthesis by a process reversible by glucose (Honig and Rabinowitz, 1966). It has been claimed to cause breakdown of messenger RNA (Wiesner *et al.*, 1965), although the interpretation of the results which led to this conclusion has been challenged (Chantrenne, 1965). It seemed important to establish that actinomycin inhibited interferon formation because of its primary effect rather than because of some secondary effect, and we therefore investigated the effect of actinomycin and of two other inhibitors of RNA synthesis on interferon production initiated by three different viruses.

The other inhibitors were 4,5,6-trichloro-1β-D-ribofuranosyl benzimidazole (TRB) and 2-mercapto-1-(β-4-pyridethyl) benzimidazole (MPB). Both compounds exerted their primary effect on RNA synthesis, although there was also some depression of DNA and of protein synthesis (Walters, Burke and Skehel, 1967). In addition, MPB caused an immediate and competitive inhibition of nucleoside phosphorylation, probably due to inhibition of the nucleoside kinase (Skehel *et al.*, 1967). Viruses which are sensitive to actinomycin (fowl plague and influenza viruses) were more sensitive to the effect of both MPB and TRB than were viruses insensitive to actinomycin (Newcastle disease, parainfluenza 1 and Semliki Forest viruses). The similarity of the antiviral spectrum to that of actinomycin suggested that MPB and TRB were possibly acting as preferential inhibitors of DNA-directed RNA synthesis. However it is unlikely that the substituted benzimidazoles were acting in exactly the same way as actinomycin, since their structures are so different. Results obtained with cell-free systems support this suggestion since DNA-directed RNA synthesis was much more sensitive to MPB than was DNA-directed DNA synthesis or RNA-directed RNA synthesis (Keir and Burke, unpublished; Martin, unpublished).

Actinomycin, TRB and MPB inhibited interferon production induced by Chikungunya virus, ultraviolet-irradiated Newcastle disease virus (u.v.-NDV) or ultraviolet-irradiated influenza virus (u.v.-MEL) at doses where their principal effect was on RNA synthesis. The structures of these compounds are so different that they are unlikely to inhibit interferon production by a common secondary process, and we concluded that DNA-directed RNA synthesis is essential for interferon production.

Several workers (Wagner, 1964; Wagner and Huang, 1966; Levy, Axelrod and Baron, 1965; Ho and Breinig, 1965) have added actinomycin

during the course of interferon formation in order to obtain data about the time of formation and the stability of the interferon messenger RNA. Interpretation assumes that the production of interferon messenger RNA is the rate-limiting process for interferon synthesis, and that actinomycin has no effect on the stability of the messenger RNA. Their results indicated that synthesis of a stable messenger RNA was complete shortly after infection. Addition of actinomycin or MPB to the Chikungunya virus-induced system gave similar results (Table II), addition one hour after infection

TABLE II

EFFECT OF ADDITION OF ACTINOMYCIN AND MPB ON INTERFERON PRODUCTION BY CHIKUNGUNYA VIRUS

Time of addition of inhibitor (hr.)	*Titre at time of addition (PDD_{50})*	*Interferon yield between time of addition and 9 hr. after addition (PDD_{50})*		
		Control	*Actinomycin (0·5 μg./ml.)*	*MPB (50 μg./ml.)*
0	0	113	<2	22
1	2	75	14	25
3	25	54	52	53
4	37	56	55	57
5	58	48	50	44
7	81	40	39	32

causing substantial inhibition of interferon production but addition three hours after infection having no effect. However when u.v.-NDV was used as an inducer, interferon formation did not become insensitive to the effects of actinomycin and MPB until 12 to 15 hours after infection (Table III). In both cases the inhibitors ceased to be effective shortly after the end

TABLE III

EFFECT OF ADDITION OF ACTINOMYCIN AND MPB ON INTERFERON PRODUCTION INDUCED BY U.V.-NDV

Time of addition of inhibitor (hr.)	*Titre at time of addition (PDD_{50})*	*Interferon yield between time of addition and 48 hr. post-infection (PDD_{50})*		
		Control	*Actinomycin (0·5 μg./ml.)*	*MPB (50 μg./ml.)*
0	0	130	3	4
8	12	81	10	12
12	25	63	25	32
16	90	52	50	50
20	103	44	44	38

of the lag period, indicating that formation of the interferon messenger RNA was the last stage in the lag period, whatever its length. In both cases the messenger RNA was stable for at least six hours. However, in the ultraviolet-irradiated influenza-virus-induced system, interferon production remained sensitive to the effects of actinomycin and TRB throughout

the period of production, the messenger RNA having a half-life of about two to four hours (Table IV). The reason for this difference is not known;

TABLE IV

EFFECT OF ADDITION OF ACTINOMYCIN AND TRB DURING INTERFERON FORMATION INDUCED BY U.V.-MEL

Time of addition of inhibitor (hr.)	*Titre at time of addition (PDD_{50})*	*Interferon yield between time of addition and 47 hr. after infection (PDD_{50})*			
		Control	*Actinomycin* (0·5 μg./ml.)	*Actinomycin* (0·06 μg./ml.)	*TRB* (10 mM)
0	0	210	<2	<2	16
18	76	140	34	34	60
22	104	84	26	26	48
26	128	56	26	26	30
30	152	37	18	21	23
35	181	26	21	18	30

it may be significant that influenza is the only actinomycin-sensitive virus that has been used for interferon production, and it is possible that the stability of the interferon messenger RNA may be dependent in some way on an early stage of virus infection, which in this case is actinomycin-sensitive.

An attempt to demonstrate the formation of the interferon messenger RNA was made, using the Chikungunya virus-induced system at 42° (Burke and Walters, 1966). Interferon-producing cells were labelled for 40 minutes with [^{3}H]uridine at hourly intervals for five hours after infection, while control cells received [^{14}C]uridine under similar conditions. The cells were mixed and successively extracted with phenol at 2°, and with phenol plus 0·5 per cent sodium dodecyl sulphate at 50°. The former extracted mainly cytoplasmic RNA (Fenwick, 1964), while the latter extract consisted mainly of rapidly labelled ribosomal RNA precursor found in the nucleus (Scherrer, Latham and Darnell, 1963). The extracts were centrifuged at 20,000 rev./min. for 16 hours on gradients of 5 to 25 per cent sucrose. Appearance of a new RNA in the interferon-producing cells would have increased the ratio of [^{3}H]/[^{14}C], but no rise could be detected. However we do not know how sensitive this method is, and it is very likely that the amount of interferon messenger RNA is below the limit of detection. A similar attempt to detect new protein formation in interferon-producing cells was made by labelling interferon-producing cells with [^{3}H]valine and control cells with [^{14}C]valine. The cells were mixed and the extracted proteins fractionated by a polyacrylamide gel electrophoresis. Again no increase in [^{3}H]/[^{14}C] could be detected. These failures are not too surprising in view of the very high biological activity of interferon,

which is at least 10^6 units/mg. protein (Fantes, 1967). The cells were producing about 45 units of interferon per hour or only about 0·05 μg. of interferon per hour. However it was possible that other proteins, as well as interferon, were produced after treatment with virus. If this was so, we were unable to detect them.

SUMMARY

Results obtained by use of Semliki Forest virus partially inactivated with hydroxylamine suggest that the complete virus nucleic acid is essential for induction of interferon formation. The pathways leading to virus production and interferon formation appear to part shortly after infection since neither virus-directed protein nor nucleic acid synthesis are necessary for interferon formation. Three inhibitors of DNA-directed RNA synthesis inhibited interferon production, showing that such synthesis was a necessary stage in interferon formation. The interferon messenger RNA was stable in two virus-induced systems but not in a third. An attempt to detect the formation of the interferon messenger RNA directly was not successful.

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