Interferon Production in Chick-Embryo Cells

THE EFFECT OF PUROMYCIN AND *p*-FLUOROPHENYLALANINE

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(Received 26 July 1965)

1. When chick-embryo cells were treated with ultraviolet-inactivated influenza virus (Melbourne strain), interferon was produced after a lag period of about 10 hr. 2. The addition of small amounts of either puromycin or *p*-fluorophenylalanine immediately after the virus inhibited the subsequent production of interferon. Both inhibitors primarily affected protein synthesis, and it is concluded that interferon production involves new protein synthesis. 3. Results obtained by the addition of either inhibitor for short periods during the lag phase demonstrated a requirement for protein synthesis during the second half of the lag phase. 4. Addition of puromycin during the course of interferon production caused almost immediate inhibition, but interferon formation became insusceptible to the action of *p*-fluorophenylalanine at about 26 hr. after infection. Possible explanations of this effect are discussed.

Interferon is an antiviral protein produced in a wide variety of animal cells in response to treatment with many viruses (Isaacs, 1963). Its production is inhibited by treatment of the cells with actinomycin, and it has been suggested that it is formed by a process involving de-repression of the host genome (Heller, 1963; Wagner, 1963). Its action is also susceptible to the effect of actinomycin and it may inhibit viral multiplication by a de-repression process (Taylor, 1965). The mechanism of production and action are of interest, not only because of its antiviral activity, but also because its production is an example of the formation of an induced protein in animal cells which can be studied in a tissue-culture system.

We have been studying the formation of interferon in chick-embryo cells after treatment with ultraviolet-inactivated influenza virus (Melbourne strain). The use of an inactivated virus, rather than an infective virus, has advantages since some of the effects due to concomitant virus growth can be avoided, and interpretation of the results is simplified. Intracellular interferon can first be detected about 10 hr. after treatment with inactivated virus. The interferon is rapidly released from the cells, and the titre in the medium increases almost linearly with time for about 40 hr., after which the rate of production gradually decreases (Burke & Buchan, 1965). The inhibition of interferon production by actinomycin (Heller, 1963;

Wagner, 1963) suggests that the synthesis of interferon is controlled by the host rather than by the viral genome. It is not known whether interferon is synthesized anew, or whether an alternative mechanism is involved, e.g. the transformation of an inactive precursor. We have therefore examined the effect of two widely studied inhibitors of protein synthesis, puromycin and FPA,† on the production of interferon. These two inhibitors were chosen because they are known to differ in their mode of action, puromycin causing premature release from the ribosomes of incomplete polypeptide chains (Allen & Zamecnik, 1962), and FPA causing its effect by incorporation into protein in place of phenylalanine, sometimes yielding an inactive protein (Munier & Cohen, 1959; Richmond, 1960, 1963). We have also investigated the effect of addition of these two inhibitors for short periods both before and after the commencement of interferon production in an attempt to obtain information about the intracellular processes on which interferon production is dependent. A brief report of this work has already been presented (Buchan & Burke, 1965).

MATERIALS AND METHODS

Media, cells and viruses. Primary chick-embryo cells were prepared and cultured in medium 199 with added calf

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† Abbreviations: FPA, *p*-fluorophenylalanine; PDD₅₀, 50% plaque-depressing dose; UV-MEL, Melbourne strain of ultraviolet-inactivated influenza virus.

serum as described by Burke & Buchan (1965), growth medium containing 7% of calf serum and maintenance medium 2% of calf serum. The calf serum used in experiments with puromycin or FPA was passed through a Sephadex G-50 column (Piez, Oyama, Levintow & Eagle, 1960) to remove free amino acids and other materials of low molecular weight. Influenza virus (strain Melbourne) was grown in the allantoic cavity of 10-day-old fertile eggs, and inactivated with ultraviolet light as described by Burke (1961). Haemagglutinin titrations were carried out as described by Lindenmann, Burke & Isaacs (1957), the haemagglutinin titre being defined as the reciprocal of that dilution giving partial agglutination. Chikungunya virus was grown in the brains of 3-day-old sucking mice. All viruses were stored at -70° .

Production of interferon. Interferon production was initiated in chick-embryo cells by treatment with UV-MEL as described by Burke & Buchan (1965). The experiments were carried out with two batches of UV-MEL, both of which produced the maximum yield of interferon when a haemagglutinin titre of 50 or higher was used. All experiments were carried out with UV-MEL of haemagglutinin titre 100, to ensure that all cultures received sufficient inactivated virus to produce maximum yields. Three Petri-dish cultures were used for each experimental point, and the fluids pooled and stored at -20° until assayed.

Interferon assay. Interferon was assayed by a plaque-inhibition technique by using Chikungunya virus to challenge (Burke & Buchan, 1965). Samples were diluted in twofold steps, with three dilutions per assay, and incubated with Melbourne virus antiserum before addition in duplicate to chick-embryo cultures. All assays also included six control cultures and suitable dilutions of an internal standard (see below). After overnight incubation at 37° , the cultures were challenged with 50–100 plaque-forming units of Chikungunya virus, and overlaid after 3 hr. Plaques were counted after 3 days, the means of duplicates taken, and the counts, expressed as percentages of the mean control count, plotted against the logarithm of the dilution. The relationship was linear when the plaque count fell between 20 and 80% of the controls, and slope of the dose-response curve was constant, both within any one experiment and also from experiment to experiment. The interferon titre was defined as the reciprocal of the dilution necessary to give a 50% depression of the control plaque count (PDD₅₀, plaque-depressing dose; Lindenmann & Gifford, 1963). All assays contained three dilutions of an internal interferon standard as a check on the reproducibility of the results. The standard had a titre of 63 ± 14 in 22 successive assays over a period of 6 months. Each sample was assayed at least twice, and the mean taken of the titres, which differed by less than 20%.

Incorporation of radioactive precursors. Duplicate cultures in glass Petri dishes were harvested at various times after addition of the radioactive precursor, and the cells washed three times with 3 ml. of cold 0.9% NaCl, three times with 3 ml. of cold 5% (w/v) trichloroacetic acid, twice with 3 ml. of cold ethanol, twice with 3 ml. of ethanol-ether (3:1, v/v) at room temperature and finally with 3 ml. of ether at room temperature. The defatted residue was dissolved by warming to 60° in 3 ml. of 98–100% formic acid, to which 6 drops of 100-volume H_2O_2 had been added, plated at infinite thinness on 2.5-cm.-diam. stainless-steel planchets, and counted by using a thin end-window Geiger-

Müller tube, at 3% efficiency, for times sufficient to reduce counting error to less than 5%. The results were corrected for background counting rate. Samples from the 'pulse' experiment with [^{14}C]valine were counted by dissolving the defatted residue in 2 ml. of 0.5 N-NaOH and adding 0.5–1.0 ml. of a dioxan-based scintillator before counting in the Packard Tri-Carb scintillation counter at 79% efficiency. Less than 10% of the radioactivity from [^{14}C]valine was found in the fraction soluble in hot 5% (w/v) trichloroacetic acid. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Materials. Racemic FPA was purchased from Calbiochem, Los Angeles, Calif., U.S.A. An aqueous solution (600 μ g./ml.) was sterilized by filtration through a Sela porcelain filter candle of maximum pore diameter 1.2μ and stored at -20° . Puromycin was purchased as the dihydrochloride from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., and sterilized and stored in the same way in FPA. Phenylalanine-free medium 199 was a gift from Glaxo Laboratories Ltd., Greenford, Middlesex. L-[U- ^{14}C]Valine (specific activity 4.8 mc/m-mole) and [2- ^{14}C]uridine (specific activity 36.9 mc/m-mole) were purchased from The Radiochemical Centre, Amersham, Bucks., and *p*-fluoro-DL-3-phenyl[1- ^{14}C]alanine (specific activity 3.2 mc/m-mole) was obtained from Calbiochem.

RESULTS

Effect of puromycin on interferon production. The effect of various concentrations of puromycin on the production of interferon is shown in Table 1. Increasing concentrations of puromycin resulted in decreasing interferon yields, a concentration of 1.1 μ g./ml. (2μ M) completely inhibiting interferon production. This concentration, which was used in all subsequent experiments, depressed the rate of protein synthesis, as measured by [^{14}C]valine incorporation, to 41% of that of controls. Puromycin has also been shown to inhibit RNA synthesis (Tamaoki & Mueller, 1963; Holland, 1963), although this effect was secondary to that on protein synthesis. The dose of puromycin used only depressed RNA synthesis, as measured by [^{14}C]uridine incorporation, by 10%. It is concluded that the primary

Table 1. *Dose of puromycin required to inhibit interferon production*

After treatment of chick cell monolayers with UV-MEL, 2 ml. of maintenance medium, containing various concentrations of puromycin, was added. At various times triplicate samples were pooled, dialysed against 0.9% NaCl and medium 199 to remove puromycin, and assayed by the plaque-inhibition method.

Concn. of puromycin (μ M)	Interferon yield (PDD ₅₀)		
	At 14 hr.	At 24 hr.	At 36 hr.
0	10	144	309
1	3	42	178
2	<1	<1	<1

effect of this dose of puromycin is on protein synthesis, and that the inhibition of interferon production is probably due to the effect of puromycin on protein synthesis.

The effect of the addition of puromycin for short periods in the lag phase was next examined, to investigate whether interferon production was dependent on protein synthesis during the lag phase. After treatment of cells with UV-MEL, they were incubated for 2, 4, 6 or 8 hr. with medium containing puromycin, which was then replaced by normal maintenance medium. Interferon production was followed over the subsequent 36 hr. with the results shown in Fig. 1. The presence of puromycin during the initial 4 hr. of the lag period did not delay the appearance of interferon (Fig. 1*a*), since the two curves are clearly identical. A similar result was obtained when puromycin was present for the initial 2 hr. and then removed. However, when puromycin was present for 6 or 8 hr. after infection, delays of about 6 and 8 hr. respectively were observed in the time required for interferon formation (Figs. 1*b* and 1*c*), although the rate of interferon production was similar in control and puromycin-treated cultures. These results show that interferon synthesis was more sensitive to puromycin added in the late, rather than the early, part of the lag phase. Since the rate of interferon production resumed at control rates after removal of puromycin, it was decided to determine the effect of preincubation with puromycin, followed by its removal, on [14 C]valine incorporation. Cultures were incubated for various times with 2 μ M-puromycin, the medium was replaced by normal maintenance medium, without washing, and the rate of valine incorporation was measured. Even after 3 hr. incubation, the rate of valine incorporation was only 82% of that of controls and the rate was depressed to 56 and 52% of that of controls after 6 or 9 hr. incubation respectively with puromycin. Interferon synthesis there-

fore appears to be more sensitive to the action of puromycin than total cellular protein synthesis, and to resume more rapidly after removal of puromycin inhibition.

The effect of the addition of puromycin during the later stages of interferon production was next investigated. At intervals after the stimulation of interferon production, the medium was removed and replaced by pre-warmed maintenance medium containing puromycin. Control cultures received similar treatment with maintenance medium. After 48 hr. the fluids were harvested and dialysed before assaying for interferon, with the results shown in Table 2. The interferon titres in the control column show the interferon yield between the time stated and 48 hr. after infection, and decrease as the time-interval decreases. Comparison with the values in the third column, which shows the interferon yield in puromycin-treated cultures, indicated that puromycin was most effective in suppressing interferon formation if added during the first 12 hr. after infection, but that only small and constant amounts of interferon were formed after the addition of the inhibitor when it was added later in the cycle. The effect of the inhibitor was not due to inhibition of interferon release since the intracellular interferon titres in inhibited cultures at 58 hr. after infection were identical with those found in control cultures. Since washing of the cultures during the production of interferon was found to decrease the final yield of interferon, it was avoided. To keep disturbance of the cells to a minimum, this experiment was also carried out by adding puromycin directly to the cultures and assaying for interferon yield at 39 hr. after infection. The results (Table 3) show that addition of the antibiotic caused immediate inhibition of interferon synthesis. The slightly lower

Fig. 1. Effect of the presence of puromycin (2 μ M) for (a) 4 hr., (b) 6 hr. and (c) 8 hr. after treatment of chick cells with UV-MEL, on the subsequent production of interferon (○), as compared with controls (●). Experimental details are given in the Materials and Methods section.

Table 2. *Effect of puromycin on interferon production*

Medium was removed from the cultures and replaced by medium containing puromycin (2 μ M) at different times throughout the production cycle of interferon. The fluids were collected at 48 hr., and dialysed before assay for the interferon formed between the time of addition of the inhibitor and 48 hr. after infection.

Time of addition of inhibitor (hr. after infection)	Interferon yield at 48 hr. (PDD ₅₀)	
	Control	Inhibitor
0	135	<1
12	78	<1
17	—	8
22	59	13
26	—	14
30	—	14
36	30	11

Table 3. *Addition of puromycin during interferon production*

Puromycin (0.2 ml. of 20 μ M) was added to chick cultures at various times after treatment with UV-MEL, and incubation continued until 39 hr. after infection. The fluids were then dialysed and assayed for interferon. Control cultures were harvested at the time at which the inhibitor was added.

Time of addition of inhibitor (hr. after infection)	Interferon yield (PDD ₅₀)	
	In control cultures at time of addition of inhibitor	In treated cultures at 39 hr. after infection
12	26	18
15	50	40
18	80	53
24	98	98
39	160	—

Table 4. *Dose of FPA required to inhibit interferon production*

Medium, containing various concentrations of FPA in phenylalanine-free medium, was added to chick-cell cultures immediately after removal of UV-MEL, control cultures being incubated with complete medium 199 (i.e. containing phenylalanine). At intervals, triplicate samples were harvested, dialysed against 0.9% NaCl and medium 199, and assayed for interferon.

Concn. of FPA (μ g./ml.)	Interferon yield (PDD ₅₀)		
	At 12 hr.	At 24 hr.	At 48 hr.
Control	12	214	309
0	<1	128	—
5	—	5	40
25	<1	<1	2
50	—	<1	<1

titres in the treated cultures were probably due to thermal inactivation of interferon.

Effect of FPA on interferon production. To determine the dose of FPA required to inhibit interferon production, chick-cell cultures, infected with UV-MEL, were incubated with 2 ml. of phenylalanine-free medium 199 containing FPA at various concentrations and the yield of interferon was measured at various times. Table 4 shows that a concentration of 25 μ g. of FPA/ml. completely depressed interferon yield, and this concentration was used in further studies. The decreased yield in phenylalanine-free medium, as compared with controls, is probably due to exhaustion of the intracellular phenylalanine pool. The action of FPA was not due to a delay in interferon release,

Table 5. *Effect of FPA on [¹⁴C]valine incorporation into chick cells*

Phenylalanine-free medium, with or without FPA, and [¹⁴C]valine (0.1 μ C/dish) were added to chick cells at the times shown. Duplicate samples were taken at hourly intervals for 5 hr., over which period incorporation was linear with time. The activity of the control cultures after 5 hr. incubation was 600 counts/min./culture.

Concn. of FPA (μ g./ml.)	Time of addition of FPA and [¹⁴ C]valine (hr. after infection)	Uptake of [¹⁴ C]valine (% of controls)
0	0	64
25	0	65
25	12	65
25	22	73
25	31	71

since the interferon yields from FPA-treated cultures at 63 and 72 hr. after infection were not significantly greater than those found at 48 hr. after infection. Nor was its inhibitory effect due to an inhibition of interferon release, since only very small amounts of intracellular interferon could be detected in FPA-treated cultures.

Measurement of the effect of FPA on protein synthesis (Table 5) showed that it depressed the rate of valine incorporation, but that this depression was due to the absence of phenylalanine from the medium rather than to the presence of FPA. Other experiments showed that the inhibitory effect was completely and rapidly reversed by the addition of normal medium, and that [¹⁴C]FPA was incorporated into cell protein. Incubation of cells with FPA for up to 8 hr. did not depress the rate of incorporation of subsequently added [¹⁴C]-uridine into RNA, showing that there was no effect of this dose of FPA on RNA synthesis.

When the cells were incubated with medium containing FPA for 3 hr. after treatment with UV-MEL, and then incubated in normal medium, the rate of subsequent interferon production was depressed, although there was no increase in the lag period (Fig. 2a). However, the presence of FPA for 6 hr. prolonged the lag period by several hours, and almost completely inhibited subsequent interferon production (Fig. 2b). Since FPA was shown to be incorporated into cell protein, the inhibitory effect of FPA is probably due to the formation of non-functional proteins, normally essential for interferon production.

The effect of the addition of FPA during the later stages of interferon production was also investigated. At intervals after treatment of

Fig. 2. Effect of the presence of FPA (25 $\mu\text{g./ml.}$) for (a) 3 hr. and (b) 6 hr. after treatment with UV-MEL on the subsequent production of interferon ($\circ$), as compared with controls ($\bullet$). Experimental details are given in the Materials and Methods section.

cultures with UV-MEL, the medium was removed and replaced by medium containing FPA. At 48 hr. after infection the fluids were harvested, dialysed and assayed for interferon, along with the fluids harvested before addition of FPA. FPA almost completely inhibited interferon production when added during the first 12 hr. after infection, but its inhibitory effect decreased over the subsequent 14 hr., until at 26 hr. after infection, and later, it produced no depression of subsequent interferon synthesis. Fig. 3 shows the decreasing susceptibility of interferon production as a function of the time of addition of FPA. The interferon yields in the control cultures are also plotted for comparison. The difference between the two curves is a measure of the increasing resistance of interferon production to FPA.

This increasing resistance could have been due to a change in permeability of the cells to FPA. However, it was found that the inhibitory effect of FPA on the rate of incorporation of [^{14}C]valine was constant, whether the inhibitor was added at 12, 22 or 31 hr. after infection (Table 5), i.e. at times when it has a very different effect on interferon synthesis. It was also found that the rate of uptake of [^{14}C]FPA was linear and independent of the time of its addition.

Attempt to measure increased protein synthesis early in interferon production. Since the experiments with puromycin and FPA had shown that protein synthesis during the latter half of the lag period was essential for interferon production, an attempt was made to detect increased protein synthesis in UV-MEL-treated cultures. Incorporation of [^{14}C]valine was measured at consecutive 1 hr. intervals until 8 hr. after infection. It was found that the specific activities of the UV-MEL-

Fig. 3. Effect of the addition of FPA (25 $\mu\text{g./ml.}$ in phenylalanine-free medium) on the subsequent production of interferon. FPA was added at various times and the interferon titre at the time of addition and at 48 hr. after infection determined in the usual way. The left-hand curve represents the sum of the interferon titres before addition of FPA and between addition of FPA and 48 hr. after infection ($\circ$ and Δ), as a percentage of the sum of the corresponding titres in the control cultures. The right-hand curve represents the interferon titre of control cultures as a percentage of the titre at 48 hr. after infection ($\bullet$ and $\blacktriangle$). Two experiments are shown with final control titres at 48 hr. after infection of 380 and 145.

treated cultures differed from those of the control cultures by less than 10%, i.e. within the limits of error of the method. Presumably the protein synthesis necessary for interferon production represents only a small fraction of that going on within the cell.

DISCUSSION

These results show that low concentrations of two inhibitors of protein synthesis, puromycin and FPA, inhibit the production of interferon in chick cells. The synthesis of interferon therefore involves new protein synthesis rather than some alternative mechanism, such as transformation of an inactive precursor. A similar conclusion has been reached by Ho & Breinig (1965) as a result of experiments with puromycin.

The presence of puromycin in the medium for the initial 2 or 4 hr. after infection did not increase the length of the lag period, nor inhibit the subsequent rate of interferon production. It appears that interferon synthesis does not require protein synthesis during the 4 hr. immediately after infection.

Zimmerman & Schafer (1960) and Wheelock (1962) have shown that the early stages in the multiplication of fowl-plague virus and Newcastle-disease virus are independent of protein synthesis. The latter author observed that adsorption and penetration of Newcastle-disease virus occurred within 1 min. of infection, and he suggested that the subsequent 2 hr. period, which was insensitive to a dose of puromycin completely blocking all protein synthesis, was required for the removal of protein and lipid from the virus particle. Similar events may be required for interferon formation after adsorption of inactivated influenza virus. When puromycin was present for 6 or 8 hr. after infection, the lag period was increased by 4–6 and 6–8 hr. respectively, but the rates of formation were not significantly different in inhibited and control cultures. These results suggest that protein synthesis is required during the entire initial 6 or 8 hr. periods, and are not consistent with those results obtained when 2 or 4 hr. incubation periods with puromycin were employed. However, removal of puromycin from chick-cell cultures did not result in a complete reversal of the inhibition of protein synthesis, the extent of reversal decreasing with increasing time of incubation with puromycin. Puromycin therefore causes some irreversible damage to the mechanism of protein synthesis, and this is possibly the explanation of the increase of the lag period by more than the expected period of 2 or 4 hr. Mueller, Kajiwara, Stubblefield & Rueckert (1962) have observed a similar effect of puromycin on protein synthesis.

The increase in the length of the lag period when puromycin was present during the first 6 or 8 hr. after infection indicates that protein synthesis is necessary during the latter half of the lag period. This protein may be interferon, although interferon was undetectable within chick cells during the lag period, or it may be an enzyme essential for interferon synthesis. An attempt to detect increased protein synthesis, as compared with controls, during the lag period was unsuccessful. The inhibitory effect produced when puromycin was added during the production of interferon demonstrates a continued requirement for protein synthesis as long as interferon is being produced, the small amounts of interferon formed after the addition of the antibiotic probably being due to the time taken for puromycin to exert its effect.

FPA exerts its inhibitory effect on protein synthesis by a mechanism different from that of puromycin. The minimum concentration of FPA that completely inhibited interferon synthesis (25 $\mu\text{g./ml.}$) produced only a small inhibition of [^{14}C]valine incorporation, and it was shown that this inhibition was due to the absence of phenylalanine from the medium rather than to the

presence of FPA. Use of [^{14}C]FPA showed that the inhibitor was incorporated into cellular protein, presumably with the formation of non-functional proteins (Scholtissek & Rott, 1961). Other workers have needed to use much higher doses of FPA to inhibit virus-induced processes (e.g. Wilcox & Ginsberg, 1963), mainly because of the use of medium containing phenylalanine, which will compete with the inhibitor. We have used phenylalanine-free medium and calf serum from which small molecules had been removed by gel filtration, and this is probably the reason for the effectiveness of the low dose employed. In contrast with puromycin, FPA had no detectable effect on RNA synthesis.

Incubation of the cells with FPA for 3 hr. immediately after treatment with UV-MEL had no effect on the length of the lag period, but depressed the rate of subsequent interferon production. This result suggests that incubation with FPA led to the formation of a non-functional form of a protein normally essential for the synthesis of interferon, and formed within 3 hr. after infection. This protein appears to be different from the one whose synthesis is inhibited by puromycin, since no requirement for protein synthesis could be demonstrated during this period by treatment with the dose of puromycin used. The presence of FPA for 6 hr. prolonged the lag period as well as depressing the final yield of interferon, and the increased lag may be due to an effect on the puromycin-sensitive protein.

When FPA was added immediately after infection with UV-MEL, or up to 12 hr. after infection, it completely inhibited interferon production. Interferon formation then became gradually less susceptible to inhibition, and at 26 hr. after infection no inhibition was detectable. This change in susceptibility was not due to a change in permeability of the cells nor to a change in the size of the phenylalanine pool, since the incorporation of [^{14}C]FPA, and the inhibitory effect of FPA on [^{14}C]valine uptake, were unchanged throughout the production period. Our explanation of this effect is that a second, FPA-sensitive, protein is synthesized until about 26 hr. after infection, and that this protein is stable and essential for interferon production. Certain proteins involved in adenovirus synthesis also display an increasing resistance to the action of FPA (Wilcox & Ginsberg, 1963). Extrapolation of the curves of Fig. 3 suggest that production of this FPA-susceptible protein begins about 8 hr. after infection or a little earlier. This protein may be identical with the puromycin-sensitive protein, first formed about 6 hr. after infection.

The results obtained by the use of these two inhibitors suggest that at least three proteins are

involved in interferon production: an FPA-sensitive protein formed within 3 hr. of treatment with UV-MEL, a puromycin-sensitive protein first detectable about 6 hr. after infection and possibly identical with a second, FPA-sensitive, protein whose formation is complete by 26 hr. after infection, and interferon itself.

We thank the Department of Scientific and Industrial Research, the Medical Research Council, Glaxo Laboratories Ltd., Imperial Chemical Industries Ltd., Pharmaceuticals Division, and the Wellcome Research Laboratories for grants which supported this work, and Mr F. Troup and Mr G. Sutherland for technical assistance.

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