INFLORESCENCE INITIATION IN *LOLIUM TEMULENTUM* L.

VI. EFFECTS OF SOME INHIBITORS OF NUCLEIC ACID, PROTEIN, AND STEROID BIOSYNTHESIS

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Summary

Single applications of 5-fluorouracil (5FU), 5-fluorodeoxyuridine (5FDU), actinomycin D, chloramphenicol, ethionine, and tris-(2-diethylaminoethyl) phosphate trihydrochloride (SK & F 7997-A_3) were made, at various times and sites, to plants of *L. temulentum* exposed to one long day, to determine when and where each had its greatest effect.

5FU and actinomycin D were most inhibitory to the induction of flowering when applied, by injection near the shoot apex, early in the daylight period following the long day. The inhibitory effect of 5FU was not reversed by applications of thymidine, but was reversed by orotic acid.

5FDU was most inhibitory when injected 2–3 days after the long day. Its inhibitory effect was not reversed by simultaneous or earlier applications of either thymidine or orotic acid.

SK & F 7997-A_3 was inhibitory to induction when applied to leaves before the critical photoperiod was reached on the long day, but was also inhibitory to induction when injected near the shoot apex, particularly when injected after the long day.

Chloramphenicol and ethionine were not inhibitory to induction when applied to the leaves, and were only slightly inhibitory when injected near the shoot apex, but without a marked time-dependence for their effect.

It is concluded that nucleic acid and protein metabolism are not directly involved in the generation of the long-day stimulus in the leaf, while steroid metabolism may be. On the other hand, ribonucleic acid synthesis at the shoot apex, on the arrival of the long-day stimulus there, is an essential component of induction in *L. temulentum*.

I. Introduction

The use of experiments in which growth substances or antimetabolites are applied at various times and sites during flower induction to get evidence of the nature of the component processes of induction has been considered in Part V of this series (Evans 1964). Salisbury (1961) has pointed out that this approach is most likely to prove of value with antimetabolites which influence only specific biochemical reactions, particularly when their effects can be reversed by simultaneous application of the corresponding metabolite. This paper reports the use of several specific antimetabolites and inhibitors in timing experiments with *Lolium temulentum* L., a long-day plant which needs exposure to only one photoperiod of about 16 hr duration for the induction of inflorescence development. The experiments have been aimed particularly at elucidation of the nature of the processes in the leaf which lead to

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the generation of the long-day stimulus, and of the processes at the shoot apex following the arrival of the stimulus.

Inhibitors of steroid biosynthesis have recently been shown (Bonner, Heftmann, and Zeevaart 1963) to suppress floral induction in the short-day plants *Xanthium pensylvanicum* and *Pharbitis nil* when applied to the leaves before, or during the early part of, the inductive dark period. One of these inhibitors, tris-(2-diethylaminoethyl) phosphate trihydrochloride (SK & F 7997-A_3), was used in the experiments reported below. Holmes and di Tullio (1962) have shown that it blocks the synthetic pathway between mevalonic acid and cholesterol, particularly at the conversion of lanosterol to zymosterol.

Changes in leaf and shoot apex proteins during induction have been shown to occur in several short-day plants (Metzner 1955; Marushige and Marushige 1962*a*; Nitsan 1962), and Collins (1960) and Marushige and Marushige (1962*b*) have shown that applications of ethionine to leaves of *Xanthium* and *Pharbitis* before, or during the early part of, the single long dark period markedly inhibited flowering. As well as interfering with protein synthesis, ethionine inhibits transmethylation (see Boll 1960), but Collins (1962) indicates that its effect on flower induction in *Xanthium* is likely to be through its effects on the incorporation of methionine into protein. Ethionine was used in some of the experiments reported below, and also chloramphenicol, which is known to be a specific inhibitor of protein synthesis in bacteria and, at much higher concentrations, in plants (Rabson and Novelli 1960; Margulies 1962).

5-Fluorouracil (5FU) inhibits flower induction in *Xanthium* when applied at the beginning of the inductive dark period (Salisbury and Bonner 1960; Bonner and Zeevaart 1962), and in *Pharbitis* even when applied at the end of the inductive dark period (Zeevaart 1962). In both of these short-day plants it inhibits the synthesis in the buds of both ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), and is incorporated into RNA. But while its effect on induction in *Xanthium* is via its effect on RNA synthesis (Bonner and Zeevaart 1962), its effect on induction in *Pharbitis* appears to be via its effect on DNA synthesis (Zeevaart 1962).

5-Fluorodeoxyuridine (5FDU), which is much more specific in action than 5FU, and primarily inhibits synthesis of DNA by blocking methylation of deoxyuridylic acid to thymidylic acid (Harbers, Chaudhuri, and Heidelberger 1959), also inhibits induction in *Xanthium* and *Pharbitis*. In *Pharbitis*, DNA synthesis in the apex appears to be an essential component of induction (Zeevaart 1962), whereas in *Xanthium* it is not (Bonner and Zeevaart 1962).

These inhibitors of RNA and DNA synthesis were used in the experiments reported below, and also actinomycin D, which specifically inhibits DNA-dependent RNA synthesis through binding to the guanine residues of DNA (Kirk 1960; Goldberg, Rabinowitz, and Reich 1962).

II. Experimental Methods

All plants were grown for at least 5 weeks in 8-hr days at 25°C/20°C, until the sixth leaf was fully expanded, before exposure to one long day. The long day consisted of 8 hr at 25°C under bright sunshine, from 8.30 a.m. to 4.30 p.m., followed by 16 hr

at 20°C under incandescent lamps which gave a light intensity of 15 f.c. at plant height. The plants were then returned to the standard short-day conditions for 3 weeks before dissection of the apices of the primary shoots.

Plants were recorded as having initiated inflorescences when they had at least reached the double ridges stage of differentiation. The stage of morphological development of each apex was recorded, together with its length above the uppermost overlapping leaf primordia. The close relation previously found (Evans 1960) between apex length and stage of morphological development was not changed to any extent by the chemical treatments reported here, and only the data on mean apex length for all plants in each treatment, excluding those in which the apices were dead at dissection, are presented.

In all experiments, all leaves except the sixth and seventh were removed at the beginning of the long day. There were 10–14 plants in each treatment.

Solutions of the antimetabolites were applied in various ways. For injections, 0·1 ml of the solution was injected by means of a hypodermic syringe into the cavity within the leaf sheaths and surrounding the shoot apex. Leaf applications were made in three ways: by immersing the sixth and seventh leaf blades in 30 ml of the solution for 10 min; as a fine spray, using 2 or 10 ml per treatment group; or by placing 0·1 ml at the base of each leaf blade. In some experiments Tween-20, at a concentration of 0·1%, was added to the antimetabolite solutions. Control plants had distilled water or 0·1% Tween solution applied in the same way as the antimetabolite solutions. In no case did Tween alone have any significant effect on flowering.

The 5FU and 5FDU samples used were provided by courtesy of Hoffmann–La Roche of Basle, Switzerland.

The actinomycin D sample was provided by courtesy of Merck, Sharp, and Dohme of Pennsylvania. It was initially dissolved in 70% ethanol, and then diluted to a concentration of 100 μg/ml in 7% ethanol. Control groups, injected with 7% ethanol alone, were included in the actinomycin experiments, but did not differ significantly from the water controls.

The sample of the steroid inhibitor, SK & F 7997-A_3, was provided by courtesy of Smith, Kline, and French laboratories, Philadelphia, and of Dr. J. A. D. Zeevaart. As soon as it was dissolved in water, the solutions were brought to about pH 7·2 by the addition of 0·1N KOH. At this pH, SK & F 7997-A_3 quickly breaks down, but the specific inhibitory effects of the compound on steroid biosynthesis and flower induction found previously are likely to be due to the breakdown products, since the solutions used by Holmes and di Tullio (1962) and Bonner, Heftmann, and Zeevaart (1963) were prepared in this way.

The sample of chloramphenicol (D-threo-*N*-dichloroacetyl-1-*p*-nitrophenyl-2-amino-1,3-propanediol) was kindly provided by Parke Davis, Detroit.

Fresh solutions of chloramphenicol, SK & F 7997-A_3, actinomycin D, ethionine, orotic acid, and thymidine were prepared for each experiment. 5FU and 5FDU solutions were used in several consecutive experiments. All solutions were stored in a refrigerator between successive applications.

III. Results

(a) *5FU Applications*

In a preliminary experiment, solutions of 5FU, at a series of concentrations, with or without Tween, were applied at noon on the day following the long day, either by injection or as drops on the leaf blades.

5FU was significantly inhibitory to induction with all methods of application but, as found by Salisbury and Bonner (1960) and Zeevaart (1962), leaf applications were less inhibitory than those near the shoot apex. Tween 20 did not increase the

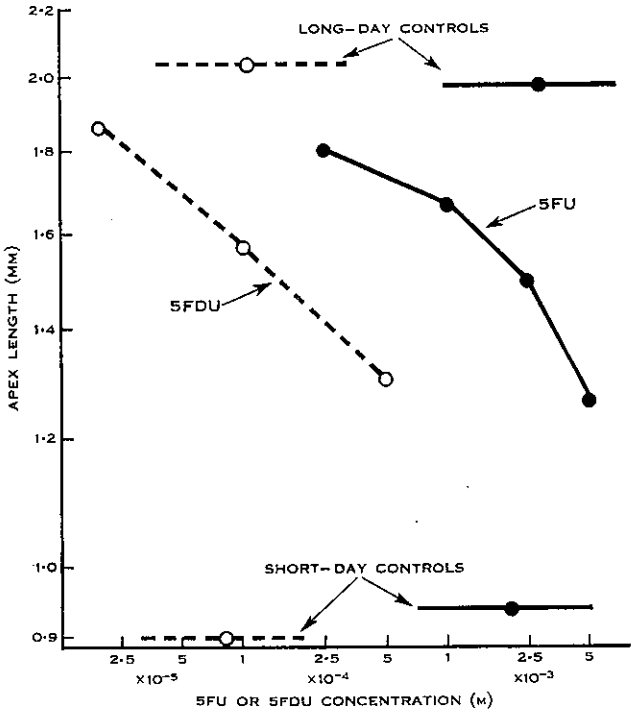

Fig. 1.—Inhibition of inflorescence development in *L. temulentum* plants exposed to one long day as a function of the concentration of 5-fluorouracil (5FU) and 5-fluorodeoxyuridine (5-FDU) solutions. 5FU was injected at noon on the day after the long-day exposure, and 5FDU at noon 2 days after the long-day exposure.

inhibitory effect of 5FU injections. All the results discussed below are for 5FU injections, without Tween. The effect of concentration of the injected 5FU may be seen in Figure 1: with solutions more concentrated than 5×10^{-3}M, most apices were dead at dissection, and even at 5×10^{-3}M, the highest concentration given in Figure 1, 10–20% of the apices were dead at dissection. Such apices are excluded from the calculations of mean apex length.

Seven experiments were carried out in which 5FU, at 5×10^{-3}M concentration, was injected at various times during long-day induction. The effect of time of

application of 5FU was similar for all experiments, and the time curves for two of them are given in Figures 2(*a*) and 2(*b*).

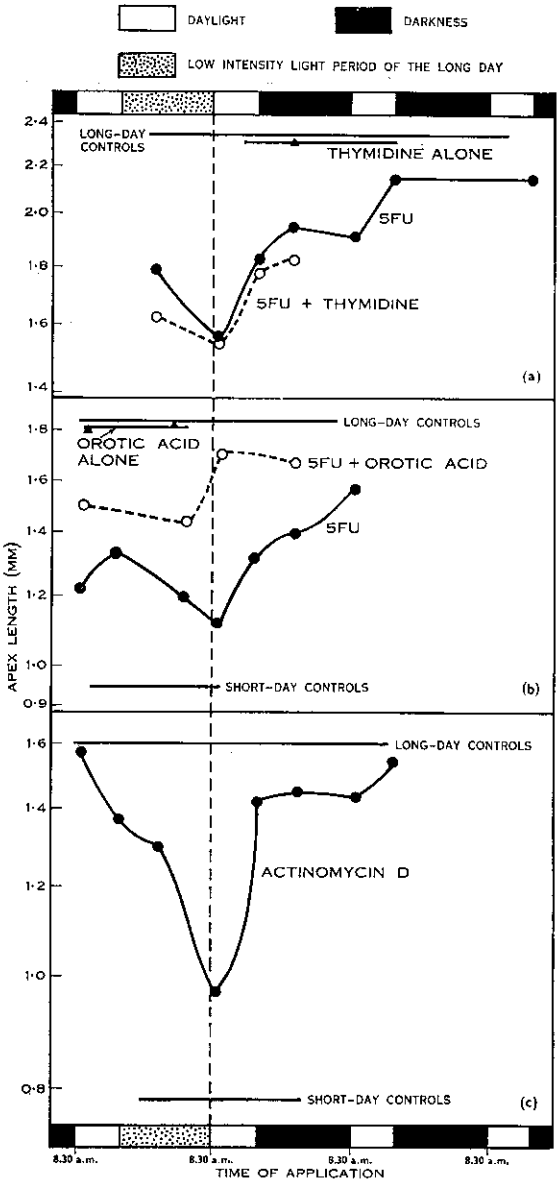

Fig. 2.—Inhibition of inflorescence development in *L. temulentum* plants exposed to one long day as a function of time of injection of 5FU (5×10^{-3}M) with or without (*a*) thymidine (5×10^{-3}M) or (*b*) orotic acid (10^{-2}M) injections 30 min previously, and (*c*) as a function of the time of injection of actinomycin D (concn. 100 μg/ml).

5FU was inhibitory to induction at all times of application, but was consistently most inhibitory when injected at 9 a.m. on the day following the long day. Table 1

gives the apex length data for these 9 a.m. injections in all experiments. In most experiments, injections at this time did not prevent inflorescence initiation in all plants, but in experiments 63 and 58 only 27% and 67% of the plants injected then subsequently initiated inflorescences. Where 5FU treatments did not prevent initiation, but reduced the rate of inflorescence development, the pattern of differentiation was normal, apart from occasional changes in the plane in which the spikelets appeared. 5FU treatment at any time had marked effects on vegetative growth of the plants, which included a great increase in tillering (even at high nodes on the primary shoot), reduction in the length of the leaf blades or sheath expanding at the time of treatment, and the production of albino zones in them, and increase in anthocyanin coloration at the base of the leaf sheaths.

TABLE 1

EFFECT OF INJECTION OF 5-FLUOROURACIL ON APEX LENGTH OF L. TEMULENTUM PLANTS AT DISSECTION 3 WEEKS LATER

5-Fluorouracil (5FU) injected at a concentration of 5×10^{-3}M at 9 a.m. on the morning following exposure to the long day. Differences significant at $P < 0{\cdot}05$ (*), $P < 0{\cdot}02$ (**), and $P < 0{\cdot}01$ (***) between long-day control and treated plants are indicated

Expt. No.	Apex Length (mm) of:		
	Short-day Control Plants	Long-day Control Plants	Long-day Plants + 5FU
51	0·99	3·28	2·34**
53	0·95	2·51	1·45***
56	0·98	2·46	1·35***
58	0·95	1·85	1·19***
61	0·88	2·35	1·57***
63	0·82	1·07	0·89*
66	0·79	1·93	1·15***

Two experiments were carried out in which, besides the treatments in which 5FU was injected at various times, thymidine solutions (also 5×10^{-3}M) were injected 30 min before the 5FU injections. The results of one of these experiments are presented in Figure 2(*a*). Thymidine alone had no significant effect on induction, but neither did it reduce the inhibitory effect of 5FU. If anything, in both experiments, thymidine slightly increased the 5FU effect.

Three similar experiments were carried out in which orotic acid solutions (10^{-2}M) were injected 30 min before the 5FU injections. The results of one of these experiments, consistent with the others, are given in Figure 2(*b*). Orotic acid alone had no significant effect on induction but, unlike thymidine, it reduced the inhibitory effect of 5FU when injected immediately before it. This "antidoting" of the 5FU inhibition by orotic acid was most marked when the orotic acid was injected at the time when 5FU was most inhibitory.

(*b*) *5FDU Applications*

5FDU, at several concentrations, was injected at noon on the second day after the long day. A majority of the apices were found to be dead at dissection following injection of the more concentrated solutions. The results at the three lowest concentrations are given in Figure 1: even with solutions of 5×10^{-4}M concentration 20–30% of the apices were dead at dissection. Some leaf applications of 5FDU were also made, as drops of a 5×10^{-4}M solution at the base of the leaf blades, and appeared to be almost as inhibitory as injections.

The effect of time of 5FDU injection was examined in two experiments, the results of which are given in Figure 3(*a*). The results of the two experiments differ so some extent, but agree in that injections at noon on the long day have least effect, while those at noon on the second or third day after the long day have the greatest effect. The time-dependence of the 5FDU effect is thus quite different from that for 5FU injections, as may be seen by comparing Figures 2(*a*), 2(*b*), and 3(*a*).

In two experiments, the inhibitory effect of 5FDU injections was not reduced by injection of either 10^{-2}M orotic acid or 5×10^{-3}M thymidine 30 min before 5FDU injection. It is particularly to be noted that the inhibitory effect of 5FDU injected at noon on the day following the long day was not reduced by simultaneous orotic acid injection.

(*c*) *Actinomycin D Applications*

Although actinomycin D is effective at extremely low concentrations in microbial systems, only concentrations as high as 50–90 μg/ml prevented differentiation in onion roots (Bal and Gross 1963). In a preliminary experiment with *L. temulentum* in which solutions of various concentrations were injected at 9 a.m. on the morning following the long day, it was found that concentrations of 33 μg/ml or less had no significant inhibitory effect on induction.

Two experiments have been carried out with injections of actinomycin D (concn. 100 μg/ml) at a series of times during long-day induction. The results of one of them are given in Figure 2(*c*). Like 5FU, actinomycin D was most inhibitory to induction when injected about 9 a.m. on the morning following the long day, at which time it prevented inflorescence initiation in 57% of the treated plants. Unlike 5FU, actinomycin D was not markedly inhibitory to induction at other times of application. This was particularly evident in the results of the other experiment in which it was inhibitory only when injected at 9 a.m. or noon on the day after the long-day exposure, and had no effect at all on induction when injected at 6 a.m. or 4 p.m. on that day.

Unlike 5FU and 5FDU, actinomycin D had no adverse effects on the growth of the treated plants, apart from a slight reduction in the length of the leaf blades and sheaths expanding at the time of treatment.

(*d*) *SK & F 7997-A_3 Applications*

In a preliminary experiment, SK & F 7997-A_3 solutions, with and without Tween-20, at concentrations of 1, 2, and 3 mg/ml, were either injected near the

shoot apex, or applied by dipping the leaves, at either noon on the long day, or noon on the following day. The addition of Tween did not significantly modify the

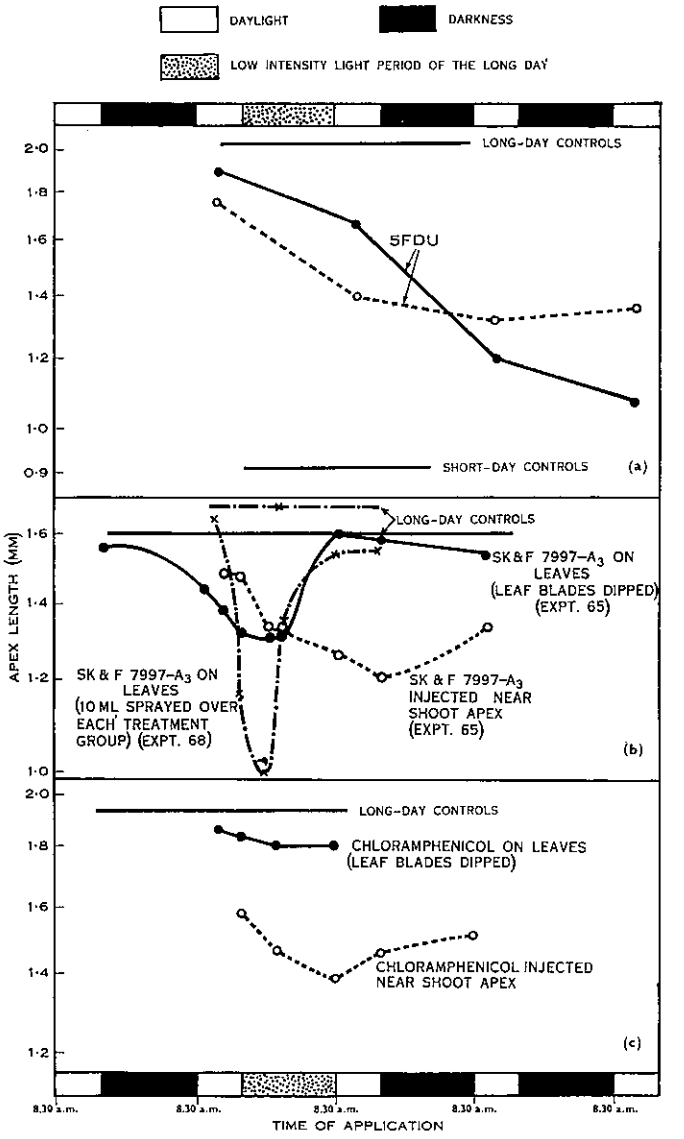

Fig. 3.—Inhibition of inflorescence development in *L. temulentum* plants exposed to one long day (*a*) as a function of the time of injection of 5FDU (5×10^{-4}M) in two experiments, (*b*) as a function of the time of application of SK & F 7997-A_3 (concn. 3 mg/ml), (*c*) as a function of the time of application of chloramphenicol (concn. 2 mg/ml).

responses, not even those to leaf applications. For both methods of application, the inhibitory effect was roughly proportional to the concentration of SK & F 7997-A_3.

Leaf applications on the long day had a significantly inhibitory effect, whereas those on the following day had none. On the other hand, injections on either day were inhibitory, particularly those on the day following the long day. This was not expected, as Bonner, Heftmann, and Zeevaart (1963) found no inhibitory effect following tip application at any time to either *Xanthium* or *Pharbitis*.

Three further experiments were carried out with applications of SK & F 7997-A_3 at various times during long-day induction, by both injection and leaf application. In one experiment a concentration of 2 mg/ml was used, in the others 3 mg/ml. Leaf applications in two experiments were by dipping the leaf blades, and in the other by spraying. The results of two experiments are given in Figure 3(*b*). Here, leaf applications made on the day before, or the days following the long day were without effect on induction, while those made up to 11 p.m. on the long day were significantly inhibitory. The inhibitory effect of SK & F 7997-A_3 was much greater, and its time-dependence more marked, in experiment 68 where heavy leaf applications were made by spraying, rather than by dipping the leaf blades. The third experiment, in which the leaf blades were dipped, yielded results similar to those of experiment 65, except that applications at 11.30 p.m. on the long day were not inhibitory, while those at 9 p.m. were most so.

From Figure 3(*b*) it may be seen that injection of SK & F 7997-A_3 near the shoot apex was also inhibitory to induction, and had a very different time-dependence for its effect, being most inhibitory when injected at 4 p.m. on the day following the long day, and still inhibitory on the next day. This finding was consistent with that of the preliminary experiment, and with that of the other timing experiment, in which injections were more inhibitory than applications by leaf dipping, most so during the day after the long day, least so during the early part of the long day.

Bonner, Heftmann, and Zeevaart (1963) found SK & F 7997-A_3 to cause no noteworthy injury to the leaves treated with it, when used at a concentration of 2 mg/ml, but with *L. temulentum* leaf applications at this concentration led to withering of the treated leaves after 2 days, although injections caused no apex deaths.

(*e*) *Chloramphenicol and Ethionine Applications*

In two experiments, chloramphenicol, at a concentration of 2 mg/ml, was either injected or applied to the leaves by dipping them at a series of times during long-day induction. The results of one of these experiments are given in Figure 3(*c*), and the results of the other were similar. There was no significantly inhibitory effect from leaf applications at any time during the long day. Injections were significantly inhibitory at all times, but without a marked time-dependence.

DL-Ethionine, at a concentration of 5×10^{-3}M, has also been applied at a series of times during long-day induction in two experiments, by injection or by spraying on leaves. In both experiments leaf applications at any time had no effect on induction, while injections had only a slight inhibitory effect, without marked time-dependence.

All applications of chloramphenicol and ethionine caused a shortening of the leaf blades and sheaths expanding at the time of treatment. Thus, their lack of effect on induction is not due to failure of uptake.

IV. Discussion

(a) *Photoperiodic Processes within the Leaves*

Zeevaart (1962) has reviewed the results of serial 5FU applications to *Xanthium*, *Pharbitis*, and *Perilla*, and concludes that production of the floral stimulus in the leaves of these short-day plants does not involve nucleic acid metabolism. Since 5FU, 5FDU, and actinomycin D all have their most inhibitory effect on induction when applied after the long day, and since 5FU is more inhibitory when injected near the shoot apex than when applied to the leaves, it seems likely that nucleic acid metabolism is not directly involved in the production of the long-day stimulus in leaves of *L. temulentum*.

Collins (1960) found ethionine to inhibit induction in *Xanthium* when applied in the early part of the one long night, and to increase the critical night length. However, of the 12 amino acid antimetabolites used by him, ethionine was the only one to have these effects, which suggests that protein synthesis may not necessarily have been involved in the generation of the floral stimulus. Chloramphenicol also had no effect on induction in *Xanthium* (Collins, Salisbury, and Ross 1963). The lack of effect on induction of applications of ethionine and chloramphenicol to leaves undergoing their long-day exposure suggests that protein synthesis is not directly involved in the production of the long-day stimulus in *L. temulentum*.

Bonner, Heftmann, and Zeevaart (1963) concluded that several of the inhibitors of steroid biosynthesis used by them, and particularly SK & F 7997-A_3, prevented flowering in *Xanthium* and *Pharbitis* by inhibiting the synthesis of the floral stimulus in their leaves. SK & F 7997-A_3 was not inhibitory when applied at any time to the growing point of either species, or when applied to the leaves after the inductive dark period. With *L. temulentum*, SK & F 7997-A_3 applied to the leaves was inhibitory to induction only when applied during the long day, before the critical photoperiod was reached. At no time did it completely suppress induction, as it did in *Xanthium* and *Pharbitis*, although heavy spray applications at 8.30 p.m. on the long day in experiment 68 prevented inflorescence initiation in 67% of the treated plants. Unlike the results with *Xanthium* and *Pharbitis*, applications near the shoot apex in *L. temulentum* were also inhibitory to induction, with a time-dependence for their effect which was quite different from that for leaf applications. Since injections of SK & F 7997-A_3 were most inhibitory when made after the long day, it cannot be argued that the injected compound is inhibiting the photoperiodic processes in the leaves following translocation to them. Nor are the effects of injections likely to be due to a general toxic action of the compound, since they show a marked time-dependence. Thus, besides inhibiting the generation of the stimulus to induction in leaves exposed to a long day, SK & F 7997-A_3 may also affect the inductive processes at the shoot apex in *L. temulentum*.

(b) *Induction of the Shoot Apex*

In both *Xanthium* and *Pharbitis*, synthesis of nucleic acids in the shoot apex appears to be an essential component of their induction. In *Pharbitis*, DNA synthesis is essential (Zeevaart 1962), while in *Xanthium* it is RNA synthesis which is so (Salis-

bury and Bonner 1960; Bonner and Zeevaart 1962). In *L. temulentum* also, it seems, from the experiments with serial applications of 5FU and actinomycin D, that nucleic acid synthesis is an essential component of induction of the shoot apex. In Part IV of this series (Evans and Wardlaw 1964), it was shown that the critical photoperiod in *L. temulentum* was reached after about 16 hr of light, and that 4 hr later sufficient long-day stimulus had moved out of the leaf blades to lead to subsequent inflorescence initiation. At the estimated rate of translocation of the stimulus, it should move through the sheath of the sixth leaf (8 cm long) and reach the shoot apex at about 9–10 a.m. on the morning following the long day. This is just the time when injections of 5FU and actinomycin D are most inhibitory to induction. It is also the time when orotic acid is most effective in antidoting the inhibitory effects of simultaneously injected 5FU.

That it is RNA synthesis at the shoot apex which is critical to induction at this time is suggested by the fact that actinomycin D, which specifically inhibits RNA synthesis, is particularly inhibitory at this time, and by the ability of orotic acid and the failure of thymidine to reduce the inhibitory effect of 5FU injections. Thymidine, if anything, enhanced the inhibitory effect of 5FU, as Salisbury and Bonner (1960) also found.

One surprising feature of both actinomycin D experiments was the relatively slight inhibition caused by applications of actinomycin D made only a few hours before the time of greatest effect. It was expected that actinomycin D, bound to apical DNA following earlier applications, would continue to block DNA-dependent RNA synthesis at the apex during induction. That it has not done so may be due, as suggested by my colleague Dr. C. I. Davern, to actinomycin D being able to bind only to DNA stripped of histone. This would imply that when the floral stimulus reaches the apex from the leaf, it causes removal of histone from the genes involved in the synthesis of the RNA which is essential to induction at this time. Actinomycin D applied before this time presumably remains bound to DNA which was free of histone at the time of application.

The time-dependence of the inhibitory effect of 5FDU injections suggests that DNA synthesis is not directly involved in the induction of the shoot apex on receipt of the long-day stimulus. However, inhibition of DNA synthesis by 5FDU in the days following induction clearly suppresses the subsequent initiation and development of inflorescences in *L. temulentum*.

V. Acknowledgments

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