INFLORESCENCE INITIATION IN *LOLIUM TEMULENTUM* L.

XI.* EARLY INCREASES IN THE INCORPORATION OF ^{32}P AND ^{35}S BY SHOOT APICES DURING INDUCTION

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Summary

There is an increase in the incorporation of ^{35}S, presumably into protein, as well as an increased incorporation of ^{32}P into nucleic acids, in shoot apices of *Lolium temulentum* plants on the morning after their exposure to 1 long day.

In six experiments, the increase in incorporation at the apex due to long-day induction ranged from 26 to 75% for ^{32}P, and from 17 to 61% for ^{35}S. In several of the experiments, the earliest increases due to induction in ^{32}P and ^{35}S incorporation into the insoluble fractions occurred in the absence of any increase in the total amounts of ^{32}P and ^{35}S in the apices. No fixed sequence of events at the apex was observed. In double-labelling experiments the increase in the incorporation of ^{35}S preceded that of ^{32}P by 20 min in one experiment, and followed it in another. Whereas the increase in ^{32}P incorporation was sustained on the second day after the long day, that of ^{35}S was not. This finding agrees with our earlier analytical results.

We conclude that an increase in protein synthesis accompanies that in RNA synthesis among the earliest effects of the floral stimulus during induction of the shoot apex.

I. Introduction

Increased synthesis of RNA in the target tissue is among the earliest effects of many hormones. In some tissues the increased synthesis involves not only messenger-RNA, but also transfer- and ribosomal-RNA (Greenman, Wicks, and Kenney 1965; Gorski and Nelson 1965).

Hormone-induced increases in protein synthesis usually follow the increases in RNA synthesis (Tata 1966; Means and Hamilton 1966). In several systems, however, a small, transient increase in protein synthesis may accompany the initial increase in RNA synthesis (Hamilton 1964; Notides and Gorski 1966). That this is independent of the increase in RNA synthesis is shown by suppression of the latter with actinomycin D; with oestrogen (Hamilton 1964; Notides and Gorski 1966), insulin (Eboué-Bonis *et al.* 1963), and growth hormone (Martin and Young 1965), treatment of the target tissue with actinomycin D prevented the hormone-induced rise in RNA synthesis, but not that in protein synthesis.

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Previous work (Rijven and Evans 1967*b*) showed a transient stimulation of nucleic acid metabolism in the shoot apex of *L. temulentum* plants at about the time of arrival of the floral stimulus, generated in the leaves by exposure to 1 long day. In this paper we present evidence for a comparable transient increase in protein metabolism, and the results of attempts to establish whether it precedes or follows the rise in nucleic acid metabolism.

II. Experimental Methods

(*a*) *Plant Growth*

Plants were grown singly in pots of perlite at 25/20°C under 8-hr days of natural light for about 5 weeks. They were then moved to artificially lit, humidity-controlled cabinets, at 25/20°C, with 8-hr days at an intensity of 3500 f.c., and a relative humidity of 85%, to adapt to these standard experimental conditions.

All lower leaves and tillers were removed, leaving only leaves 5, 6, 7, and 8 (expanding) on the main shoot. The most uniform plants were then selected and randomized between two cabinets, one kept on short-day conditions while the other provided exposure to 1 long day, by extension of the 8-hr period of high intensity light with incandescent light of 50 f.c. intensity for 16 hr.

(*b*) *Isotope Application*

At various times on the day after the long day (day II), lots of 10 plants were taken alternately from the long- and short-day cabinets for treatment. Full details of the treatment methods have been given by Rijven and Evans (1967*b*). Defined areas near the base of the blades of leaves 6 and 7 were rubbed with a 1% solution of Tween 20, 10 μl of an adjuvant solution containing 50 mM citric acid and 2% glycerol were added, and finally 10 μl of carrier-free [^{32}P]phosphate or [^{35}S]sulphate solution. The activity of these solutions was usually 1–2 mc per millilitre. The plants were then returned to the cabinets until harvest.

In the early experiments ^{32}P and ^{35}S were applied to separate lots of plants. In later experiments on the timing of changes in incorporation of ^{32}P *vis-à-vis* that of ^{35}S, the two isotopes were applied to separate areas on the same leaves.

To increase the speed and reduce the labour of isotope application, trials were made with cut shoots. It was found that these responded to long-day induction almost as well as intact plants. Their apices incorporated ^{32}P from a solution around the base of the cut shoots, and showed an increase in incorporation on day II due to long-day treatment. In fact, incorporation of ^{32}P by apices was more rapid in cut shoots than following leaf applications. With ^{35}S, however, cut shoots were much less satisfactory than leaf applications. Incorporation was slower and more irregular, and comprised a smaller proportion of the total ^{35}S activity in apices than it did following leaf applications for the same period (6·0%; c.f. 11·2% in one experiment). These differences may reflect the need for sulphate reduction in leaves prior to transport and incorporation in the apex.

(*c*) *Extraction Procedures*

After an interval of 3–4½ hr, the groups of 10 plants were harvested in the order of treatment, their shoot apices dissected, and cut across the base above the youngest leaf primordium showing upwards growth. They were then extracted by the procedure followed by Rijven and Evans (1967*b*) in their later experiments. The apices were dried and weighed on a Cahn electrobalance. Individual apices were usually about 2 μg in weight. The activity remaining in the extracted apices is referred to as incorporated activity, and is expressed as counts per minute per microgram of residual dry weight. Significant changes in dry weight of apices due to long-day treatment do not appear as early as day II (Rijven and Evans 1967*b*; Table 2). The extracts of the apices from each treatment were bulked and their activity also estimated in order that total apical activity, as well as incorporated activity, could be determined for each treatment.

(*d*) *Counting*

In the early experiments in which ^{32}P and ^{35}S were applied to separate lots of plants, the activities of the apices, their extracts, and of the solutions applied to the plants, were determined with a gas-flow, end-window Geiger–Müller tube.

In the later experiments where ^{32}P and ^{35}S were applied to the same plants, liquid-scintillation counting was used to differentiate the disintegrations of the two isotopes. After weighing, the apices were digested in 1 ml of Packard hydroxide of Hyamine, at 50°C for 1 hr. Counting of the activity incorporated in apices was found to be irregular without such preliminary digestion. 6 ml of a solution of 2,5-diphenyloxazole in toluene was added as scintillant, and counting done on a Packard Tri-Carb liquid-scintillation spectrometer. For the extracts, aliquots of up to 100 μl were added to 7 ml of a solution of naphthalene, 2,5-diphenyloxazole, and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in dioxane, toluene, and ethanol.

One channel of the spectrometer was on a low-gain setting and recorded only ^{32}P disintegrations with high efficiency. The other two channels were on higher-gain settings which recorded disintegrations of ^{35}S with high efficiency, and those of ^{32}P with about one-third maximum efficiency. Channel conversion factors for ^{32}P and ^{35}S were determined from appropriate blanks in each experiment, and were used to make two independent estimates of ^{35}S activity in each sample. These usually agreed to within 1–2%.

III. Results

Results for three experiments in which ^{32}P and ^{35}S were applied to separate lots of plants are presented in Table 1. For all pairs of treatments in all experiments, exposure of plants to a long day has resulted in an increased incorporation of ^{32}P and of ^{35}S during the following morning. The average increase in ^{32}P incorporation over the three experiments was 44%, compared with an average increase of 35% in our earlier experiments over 3 hr (Rijven and Evans 1967*b*). The average increase in ^{35}S incorporation was 34%.

The magnitude of the increase varied somewhat from one experiment to another, and from one treatment to another. For both ^{32}P and ^{35}S incorporation, the increases tended to be smaller for the late-morning applications. For both isotopes the amount incorporated by the apex represented only about 1×10^{-6} of the activity applied to each plant, and usually between 10 and 15% of the total activity in each apex.

TABLE 1

EFFECT OF A LONG-DAY TREATMENT ON THE AMOUNTS OF ^{32}P AND ^{35}S INCORPORATED BY, OR PRESENT IN, SHOOT APICES ON THE MORNING AFTER THE LONG DAY, $4\frac{1}{2}$ HR AFTER LEAF APPLICATION

Isotope Treatment	Time of Application to Leaf (a.m.)	Incorporated Activity (counts/min/μg)		Total Activity (counts/min/10 apices)	
		Short Day	Long Day	Short Day	Long Day
		Experiment 128			
^{32}P	9·00— 9·20	5·1	7·1	773	797
	9·40—10·00	5·3	7·1	845	1139
	10·20—10·40	6·0	7·1	908	1072
^{35}S	9·20— 9·40	1·4	2·2	359	400
	10·00—10·20	1·5	2·3	332	525
	10·40—11·00	2·0	2·8	348	409
		Experiment 131			
^{32}P	9·30— 9·50	5·6	10·1	1081	1590
	10·10—10·30	8·2	14·1	1214	1910
	10·50—11·10	5·7	9·7	1067	1435
^{35}S	9·50—10·10	2·1	2·9	387	447
	10·30—10·50	3·1	3·6	428	530
	11·10—11·30	2·8	3·8	381	444
		Experiment 135			
^{32}P	9·00— 9·20	3·5	4·8	735	826
	9·40—10·00	2·9	3·7	548	771
	10·20—10·40	3·0	3·1	533	468
^{35}S	9·20— 9·40	2·2	2·6	882	926
	10·00—10·20	2·1	3·0	745	899
	10·40—11·00	2·9	3·3	872	912

With the later isotope applications, increased incorporation by the long-day apices was usually accompanied by a substantial increase in their total activity. However, with the earliest applications of isotope there were marked increases in incorporation by long-day apices despite only slight increases in total activity. In experiment 128, for example, the increases in incorporation of ^{32}P and ^{35}S were 40 and 57% respectively for applications made between 9 and 9.40 a.m., whereas total activities increased only 3 and 11% respectively. The average increases in total apex activity due to long-day treatment over the three experiments were 27% for ^{32}P and 19% for ^{35}S.

Table 2 presents the average increases due to long-day treatment in ^{32}P and ^{35}S incorporation by apices on day II for four experiments, and the peak increases found in two other experiments (136 and 137). The magnitude of the increases varied considerably between experiments, the increase in incorporation of ^{32}P being rather more than that of ^{35}S, except in experiment 128.

The effect of long-day treatment on day III incorporation of ^{32}P and ^{35}S by apices was examined in four experiments, the results of which are also summarized in Table 2. The increased incorporation of ^{32}P due to long-day treatment was sustained in day III apices. The increase was less than that found in earlier

TABLE 2

PERCENTAGE INCREASE IN THE INCORPORATION OF ^{32}P AND ^{35}S (AS COUNTS/MIN/μG) BY SHOOT APICES OF *L. TEMULENTUM* DUE TO EXPOSURE OF PLANTS TO CONTINUOUS LIGHT ON DAY I

^{32}P and ^{35}S applied to separate lots of plants in experiments 125–135, and to the same plants in experiments 136 and 137

Experiment No.	Time (hr) from Isotope Application to Harvest	Day II		Day III	
		^{32}P	^{35}S	^{32}P	^{35}S
125	3	53	17		
128	4½	31	50		−10*
131	4½	74	26	27	−5
135	4½	26	25	17	9
136	3½	63	27	16	−5
137	3½	75	61		

* With cut shoots.

experiments (Rijven and Evans 1967*b*), possibly because the present experiments were carried out on plants grown during the winter. In contrast to the results with ^{32}P, day III incorporation of ^{35}S by apices was generally lower in the plants exposed to a long day. The increase in experiment 135 is due to anomalously low ^{35}S incorporation in one of the three short-day control treatments.

Three experiments were carried out in which both ^{32}P and ^{35}S were applied to alternate lots of control and long-day-treated plants, in an attempt to determine whether the increase in ^{35}S-incorporation on day II preceded or followed the increase in ^{32}P incorporation. To increase our time resolution, the period between application and harvest was shortened to 3½ hr. The results of two such experiments are shown in Figure 1.

In experiment 136 [Fig. 1(*a*)] there was a rise in ^{32}P incorporation from the earliest times of application. Initially, incorporation of ^{32}P by apices of the long-day plants was similar to that by the short-day controls, but the rise in incorporation was sustained longer, reaching a peak for the applications at 9.30 a.m. For these, incorporation of ^{32}P was 63% higher than that in short-day apices. This increase in incorporation due to long-day treatment was not due to a higher total ^{32}P activity in the apices, as may be seen from Figure 1(*c*).

The time trend in ^{35}S incorporation by short-day apices in experiment 136 differed from that for ^{32}P incorporation. There was an initial rise in incorporation, as with ^{32}P, and a further rise for applications between 10 a.m. and 11 a.m. Long-day apices showed an increased incorporation of ^{35}S even in the earliest treatments.

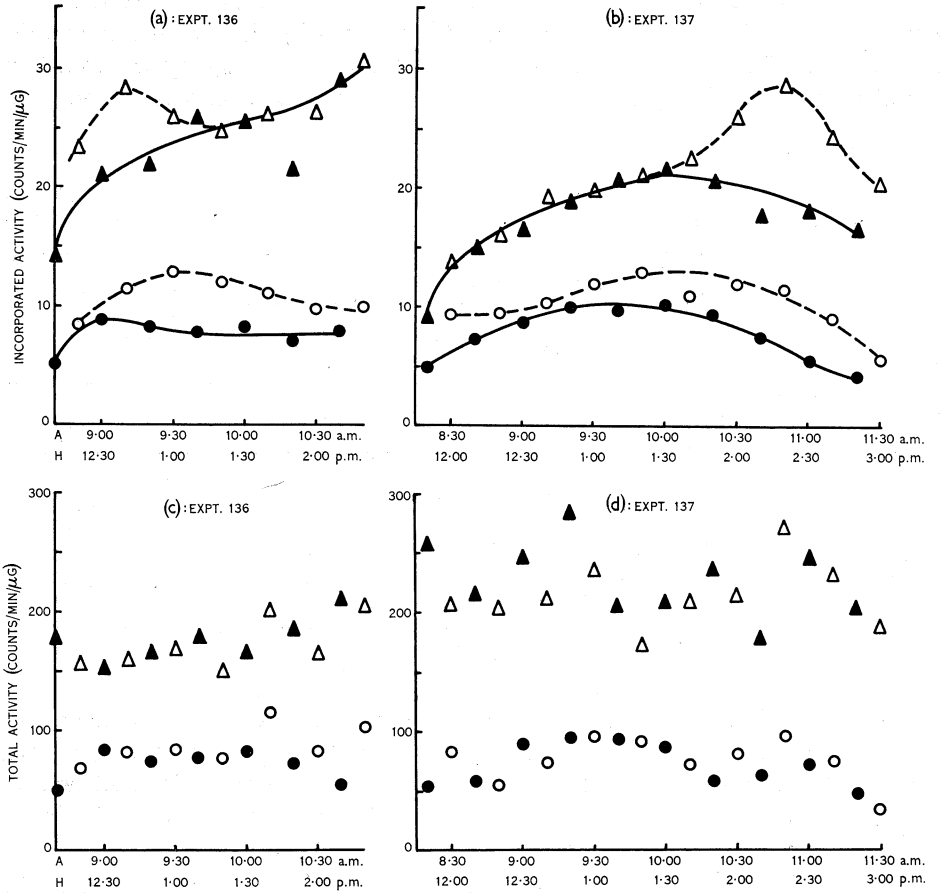

Fig. 1.—Variation with time of application and harvest on day II of the total activity (*c*, *d*) and of the amount incorporated (*a*, *b*) of ^{32}P (●, ○) and ^{35}S (▲, △) in shoot apices of control short-day plants, and of those exposed to 1 long day. Plants were harvested $3\frac{1}{2}$ hr after isotope application. ●, ▲ Short-day controls. ○, △ Plants exposed to 1 long day. *A*, time of isotope application. *H*, time of harvest.

This increase reached a peak of 27% for the application at 9.10 a.m., and then disappeared. The results for total ^{35}S activity in the apices [Fig. 1(*c*)] indicate that increased incorporation in the early long-day apices was not due to increased total ^{35}S activity.

In experiment 137 [Fig. 1(*b*)], apices from long-day plants showed an increased incorporation of ^{32}P at all times of application, the increase being greatest (75%)

for applications at 10.50 a.m. No difference in ^{35}S incorporation by long- and short-day apices was evident for applications up to 10 a.m. after which there was a marked transient increase in incorporation by long-day apices, reaching a peak 61% higher than in the short-day controls for applications at 10.50 a.m. In this experiment also there was no obvious difference between the control and the long-day apices in their total ^{32}P or ^{35}S activity at any time [Fig. 1(*d*)].

In the third experiment with simultaneous applications of ^{32}P and ^{35}S, increased incorporation of ^{32}P by the long-day apices was evident at all times of application, the increase ranging from 46 to 58%. However, no increase in ^{35}S incorporation due to long-day treatment was evident at any time of application. This was the only experiment, of seven, in which increased ^{35}S incorporation by long-day apices was not found. A possible explanation is that our earliest isotope applications, at 8.30 a.m. on day II, were too late to detect an increase in ^{35}S incorporation, since a 58% increase in ^{32}P incorporation by long-day apices was already evident. Variation between experiments in the time of translocation of the floral stimulus has already been shown (Evans and Wardlaw 1964).

IV. Discussion

These experiments have shown that besides a transient increase in the incorporation of ^{32}P by apices of plants exposed to a long day the day before, there is a comparable increase in the incorporation of ^{35}S, presumably into protein. Such increases could be due to:

(1) A direct after-effect of the long-day plants having been in low intensity light (50 f.c.) during the preceding night while the controls were in darkness.

(2) Increased translocation of the isotopes to the shoot apices of plants exposed to the long day, resulting in increased specific activity of their phosphate and sulphur pools.

(3) A specific inductive effect of the long-day stimulus to flowering on arrival at the shoot apex.

With regard to (1), an after-effect of the low intensity light, such an effect could be expected to be greatest for the earliest isotope applications. All plants were in light of 3500 f.c. intensity between isotope application and harvest, and a direct after-effect of light of only 50 f.c. intensity is unlikely to be marked when all plants have had several hours in high intensity light. The fact that increased incorporation was not always evident after the earliest applications, but only later, as in experiments 136 for ^{32}P and 137 for ^{35}S, argues against this explanation.

As for (2), it is clear from the results presented above that exposure to a long day frequently resulted in an increased total ^{32}P or ^{35}S activity in the apices. In nearly all cases, however, the percentage increase in incorporation of both isotopes was much higher than the increases in total activity in the apices. For the experiments presented in Table 1, the average increases in incorporation were 44% for ^{32}P and 34% for ^{35}S, while the average increases in total activity were 27 and 19% respectively. More cogently, marked increases in incorporation of both isotopes were often

found after early applications, in the absence of any increase in total activity of the apices (e.g. Fig. 1).

We conclude, therefore, that the increases in both ^{32}P and ^{35}S incorporation in apices of plants exposed to a long day are probably due to a specific effect of the floral stimulus. Earlier work, examining the time when this stimulus is exported from the leaves and the velocity of its translocation, suggested that the long-day stimulus probably reaches the shoot apex late on the morning of day II (Evans and Wardlaw 1966). With early morning applications of isotope and a $4\frac{1}{2}$-hr interval between application and harvest, all treatments could be expected to span the time of arrival of the stimulus at the apex, and show increased incorporation of both isotopes. This is the case for the results in Table 1.

With even earlier applications of isotope, and only $3\frac{1}{2}$ hr to harvest, plants could be harvested before induction of the apex occurred. There would then be little difference in incorporation for the earliest applications, but one would become evident as the time between application and harvest spanned the time of arrival of the floral stimulus. If the initial effect of the stimulus on RNA and protein synthesis was transient, later isotope applications would show smaller increases in incorporation due to long-day treatment. This is so for the results in Figure 1, particularly for applications of ^{35}S.

Further evidence that the increases in isotope incorporation are a specific effect of floral induction comes from experiments on the localization of these increases as revealed by autoradiography. While some increase in ^{32}P and ^{35}S incorporation occurred throughout the apices, by far the most striking increases were confined to the pockets of cells which give rise to the spikelet primordia (Knox and Evans unpublished data).

Our experiments do not indicate any fixed sequence between the increases in ^{32}P and ^{35}S incorporation. In experiment 136 the increase in ^{35}S incorporation preceded that in ^{32}P incorporation by about 20 min, whereas in experiment 137 incorporation of ^{32}P rose before that of ^{35}S. Similarly, increased uptake of both isotopes by long-day apices sometimes accompanied and sometimes followed the increases in incorporation. As in much of the work with animal hormones, we have early increases in isotope uptake, in nucleic acid metabolism, and in protein metabolism in the target tissues, but cannot ascribe primacy to any one of them.

The different sequence of effects in different experiments may reflect differences in the time and rate of arrival of the floral stimulus. RNA and protein synthesis in the apices may have different diurnal trends, and expression of the effect of the floral stimulus could depend on when it arrives in relation to these trends.

Since there is no fixed sequence for the increases in RNA and protein metabolism, they may be independent secondary effects of some primary change wrought by the floral stimulus. In this connection it is noteworthy that the relative increases in ^{32}P and ^{35}S incorporation by the apices are of similar magnitude. The marked inhibition of floral induction by injections of actinomycin D early on day II (Evans 1964) suggests that RNA synthesis at that time is necessary for induction. Whether protein synthesis at that time is also essential for induction in *L. temulentum* is still not clear.

Injections of ethionine early on day II had little effect on induction; injections of chloramphenicol, *p*-fluorophenylalanine, or cycloheximide were inhibitory, but not much more so than injections at other times, except in the case of cycloheximide (Evans 1964 and unpublished data). In the long-day plant *Lemna gibba* (Umemura and Oota 1965), and the short-day plants *Xanthium pennsylvanicum* (Collins, Salisbury, and Ross 1963), and *Pharbitis Nil* (Marushige and Marushige 1962), ethionine was inhibitory to induction, but also to growth. The time course of inhibition was studied with *Xanthium*, for which both ethionine and *p*-fluorophenylalanine were inhibitory only when applied before the critical dark period. Thus, there is no clear evidence in these species of a requirement for protein synthesis in the shoot apex during induction.

One further point requires discussion. Our earlier chemical analyses of apices of *L. temulentum* (Rijven and Evans 1967*a*) showed that protein content increased less than the other apical components between day I and day IV. Yet here we have consistently found evidence of increased incorporation of ^{35}S into protein on day II. However, this increase was more transient than that for ^{32}P incorporation on day II, and whereas increased ^{32}P incorporation by long-day plants was also evident on day III, ^{35}S incorporation on day III was often slightly lower than in the short-day controls. Thus, there is probably no discrepancy between these results and our earlier analyses.

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