

Control of Flowering in *Bougainvillea* "San Diego Red"

METABOLISM OF BENZYLADENINE AND THE ACTION OF GIBBERELIC ACID IN RELATION TO SHORT DAY INDUCTION

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ABSTRACT

Benzyladenine (BA) and short day (SD) induction promote and gibberellic acid (GA) inhibits flowering in *Bougainvillea* "San Diego Red." GA is an overriding vegetative signal maintaining plants in a vegetative state even when BA is applied in SD conditions. SD promotes a more rapid conversion of BA to the ribotide and other "polar derivatives" (containing adenine derivatives). This effect of SD on BA metabolism is seen in root, stem, and apical bud tissues and is completely prevented by prior or simultaneous application of GA. GA treatment reduces the rate of polar derivative formation to that found in plants held in long days. The working hypothesis is that SD promotes flowering in *Bougainvillea* owing to reduced transport of gibberellins from leaves to roots and apical buds permitting metabolism of cytokinin, and perhaps other purine bases, to more polar forms that are more readily translocated and active in promoting reproductive development of the inflorescence axes.

SD¹ induction and BA promote primordial initiation and development in the inflorescence axes of *Bougainvillea* "San Diego Red" (17, 19). GA inhibits primordial initiation regardless of day length or cytokinin treatment (5) and is an overriding vegetative signal not only in *Bougainvillea* but in many other polycarpic plants as well (15). The basic objectives of the studies with *Bougainvillea* are to uncover the nature of day length, BA, and GA interactions and suggest hypotheses concerning the roles of naturally occurring gibberellins and cytokinins in the control of reproductive development.

MATERIALS AND METHODS

Rooted cuttings of *Bougainvillea* "San Diego Red" were cultured to the 15-mature-leaf stage in greenhouses in LD essentially as described previously (19). For treatment of root systems, plants were cultured in an aerated nutrient solution. LD and SD treatments of plants were in 21 C fluorescent/incandescent lighted chambers (light flux, 73 w m⁻²). The plants received 8 h of this light daily and were then transferred to 21 C dark chambers for 16 h to give SD induction. LD conditions were established by interrupting the middle of the 16-h dark period with 4 h of incandescent light giving 50 to 100 lux at plant tops. To reduce the amount of plant material to be assayed for activity, plants were partially defoliated prior to treatment so that they had six young leaves unfolded from the terminal bud and three fully

expanded leaves at the base of the stem. Earlier experiments had demonstrated that plants so treated were fully capable of responding to SD induction.

[8-¹⁴C]BA (Amersham Radiochemical Centre), 1.34 mCi/mmol, was used in all experiments. Essentially all of the ¹⁴C in the samples used co-chromatographed with unlabeled BA (Sigma Chemical Co.) and no other UV absorbing materials appeared. Precoated silica gel thin layer plates (Merck Silica Gel 60) and one solvent system (A), chloroform-methanol-water (10:1.5:0.15, v/v/v) were used for all basic separations. In this system R_f values for important BA metabolites were: BA, 0.69; BAR, 0.46; adenine, 0.23; AMP, ATP, and other phosphorylated derivations, 0.02.

The basal ends of petioles of leaves or of the stems or the root system itself were immersed in shallow layers of one-fourth-strength Hoagland solution to which [¹⁴C]BA was added. GA was added dropwise, 10 μl of a 10 mg/l solution to the apical bud or sprayed (1 mg/l solution) on the entire plant on the 5th day of SD induction. Before extraction, to analyze for ¹⁴C, the portion of the stem and leaf tissues that was immersed in the solution was excised and discarded. Root tissues which were immersed in the solution of one-fourth strength Hoagland were rinsed in 3 volumes of nutrient solution prior to extraction.

RESULTS

IDENTIFICATION OF METABOLITES

For one experiment with root tissues that were treated with [¹⁴C]BA, the ¹⁴C from the R_f values associated with suspected BA and BAR were eluted from TLC plates with 80% methanol, injected on a HPLC (Tracor model 970) 25-cm Partisil PXS-10/25-ODS column, and eluted with 50% methanol at a flow rate of 1 ml/min. Detection of unlabeled BA and BAR (Sigma Chemical Co.) at 271 nm (Tracor variable wavelength detector) was at the same retention times as the ¹⁴C-labeled spots, determined by liquid scintillation counting (Fig. 1). Approximately 80% of the suspected [¹⁴C]BA added was recovered in the elution volume for authentic BA and 95% of the suspected [¹⁴C]BAR was recovered in the elution volume for authentic BAR.

One of the important BA metabolites found in plant tissues is the BA-7-glucoside (4, 9). Employing a solvent system, chloroform-ethanol (9:1), which separates clearly BAR (R_f 0.2-0.3) from the BA-7-glucoside (R_f 0.7-0.8), no evidence was found for the latter. Also, no ¹⁴C at the R_f value expected for adenine was found following 24- or 48-h incubation, which suggests that the benzyl group remains intact, or that it is released only after the BA is derivatized, or that free adenine is rapidly metabolized. Labeled compounds remaining close to the origin after chromatography in solvent system A were in part phosphorylated derivatives of BA as shown by phosphodiesterase digestions of eluates. The digestions were with 10 unit/ml at pH 8.8 of phosphodiesterase I (snake

¹ Abbreviations: SD: Short day; LD: Long day; BAR: N⁶-benzyladenosine (or, benzyladenine riboside); HPLC: High performance liquid chromatography.

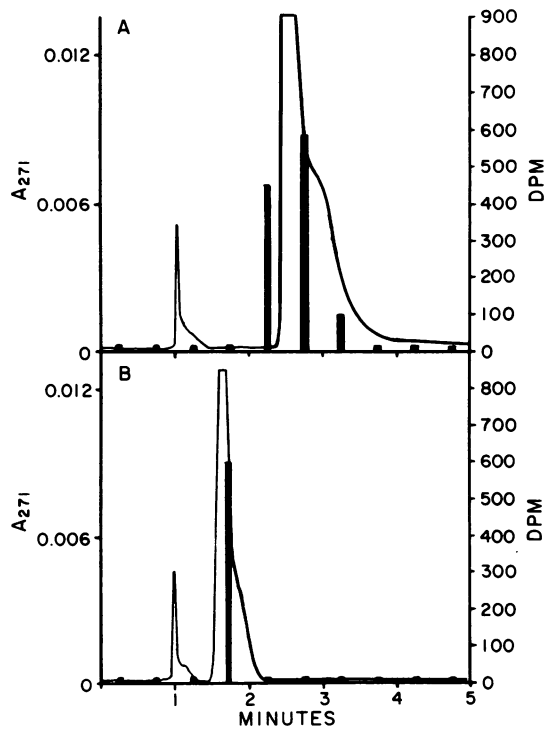


FIG. 1. HPLC analysis of R_f values from TLC corresponding to BA (A) and BAR (B). Column; 25 cm Partisil PXS-10/25-ODS; isocratic elution with 50% methanol; flow rate ml/min; UV detector at 271 nm. A trace (—); bars represent ^{14}C , in dpm. First peak in A trace at 4 min is solvent front.

venom, Sigma Chemical Co.) for 22 h at 37 C and of 1 unit/ml of phosphodiesterase II (bovine, Sigma Chemical Co.) at pH 6.9 for 22 h at 37 C. Rechromatography of the digestion mixture revealed significant activity at R_f values corresponding to BA and BAR (Fig. 2). This suggests that BA is phosphorylated directly on the benzyl or purine moiety and also that BAR is converted to the ribotide. An alkaline phosphatase digestion appeared to be almost without effect. Since much of the ^{14}C remains near the origin subsequent to incubation, we conclude that compounds other than phosphorylated BA derivatives are present and/or the enzymic treatments are incapable of hydrolyzing the intermediates. Further research is required to resolve the nature of the ^{14}C compounds in this important peak, which probably contains adenosine-5'-phosphates (9). In the paper this peak shall be referred to as the "polar" fraction.

The [^{14}C]BA incubation medium was analyzed in all cases and no evidence was found for significant metabolism after 24 to 48 h in the medium used for incubation of root or shoot tissues. That is, essentially all of the ^{14}C recovered from the medium is associated with the peak for BA.

BA METABOLISM IN ROOT TISSUES

Following a 24-h incubation of root tissues attached to plants that had received 6 SD or LD only, there are two major peaks of ^{14}C in the SD and one major and minor peak in the LD plants (Fig. 3, A and B). BA appears at R_f 0.7 and the large peak found near the origin in the extracts of the SD plants is composed in part of phosphorylated derivatives. BAR was present in very small amounts in both extracts. Part of the BA peak may represent that portion not readily removed by the rinsing procedures employed.

The roots of SD-induced plants are significantly more active in forming the more polar derivatives. The absence of BAR in these roots suggests that the ribotides are formed directly by combination of BA with a ribosylphosphate. Since very little ^{14}C moved

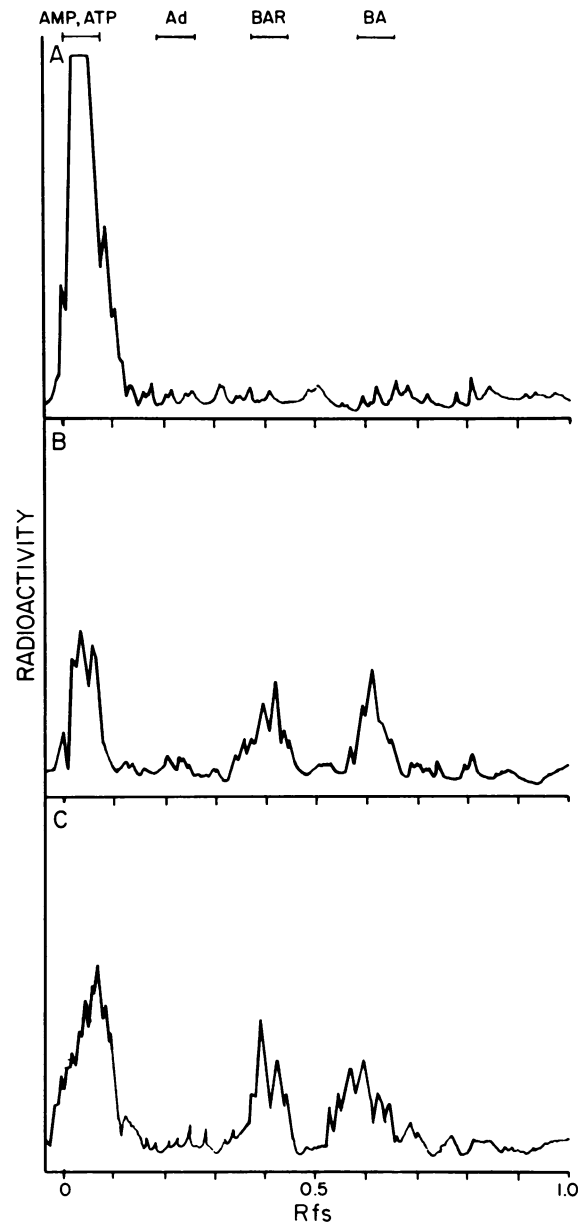


FIG. 2. Traces of ^{14}C scans of TLC of ^{14}C eluted from R_f 0 to 0.2, incubated at 37 C in phosphodiesterase I (B) or phosphodiesterase II (C), reappplied to silica gel TLC and developed in solvent system A. Uppermost trace (A) is for the control eluate that was incubated in boiled enzyme.

into the shoot system, never more than 3 to 6%, no further analysis of shoot tissue extracts was attempted.

GA INFLUENCE ON BA METABOLISM IN ROOT SYSTEM

Root systems of SD-induced intact plants were incubated in [^{14}C]BA for 24 h; in one case GA at 1 mg/l was added to the incubation solution and in another the shoot system was sprayed with 1 mg/l BA 24 h earlier. TLC analyses of extracts of the root systems reveal that in both GA treatments very little BA is metabolized to the phosphorylated derivatives (Fig. 3C), particularly in comparison to the SD group (Fig. 3A). Since there is no difference in BA metabolism, whether the roots are incubated in GA or whether GA is applied to the shoot system, only the data for one type of GA treatment are given.

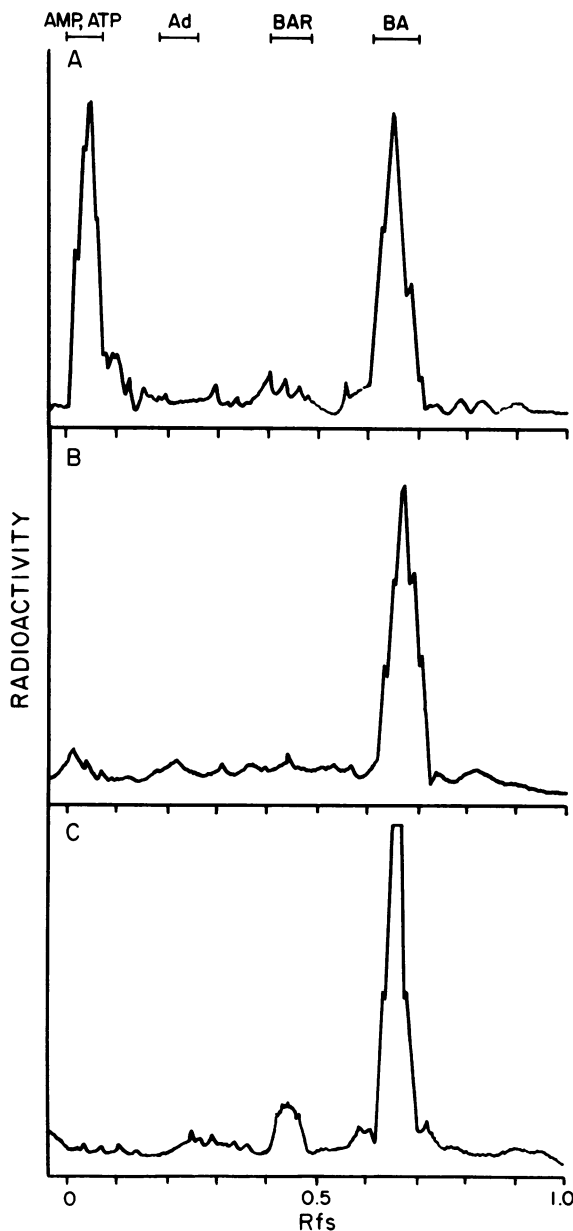


FIG. 3. Traces of ^{14}C scans of TLC of extracts of root system of SD (A), LD (B), SD + GA (C) plants 24 h after incubation with [^{14}C]BA. GA (1 mg/l) was spray-applied to the shoot system 24 h before incubation with [^{14}C]BA; the same results were obtained when the root systems were incubated with GA (10 mg/l) added to the medium simultaneously with [^{14}C]BA.

METABOLISM IN SHOOT SYSTEM

Leafy Stem Cuttings. Shoot systems, detached from their roots, following SD induction or in LD only, were treated directly to discover whether they were capable of independent metabolism of BA. Stem bases were immersed in [^{14}C]BA solutions and the shoot tissues, divided into parts as before (except that the immersed portion of the stem was discarded), harvested, and extracted 24 h after treatment. Considerably more ^{14}C is found in the shoot systems in the SD-induced plants than in the LD or SD + GA shoot systems (Table I). Radiochromatographic scans for extracts of stem tissues (Fig. 4) show relatively more BAR and polar derivatives in the SD than in the LD or in the SD + GA. The presence of BAR in these tissues is a sharp departure from the findings for the root system. Free BA does not persist in stem

tissues in substantial quantities. It is likely then that the xylem-mobile forms of BA are BAR and the more polar derivatives.

Analyses of extracts of mature and young leaves attached to stem tissues reveal most activity in the BAR region (Fig. 5, A and B) which suggests that BAR is the major xylem-mobile form and/or that leaves rapidly convert the more polar derivatives to BAR. There are no qualitative differences in BA derivatives apparent in the extracts of leaves from LD, SD or SD + GA plants. It appears then that SD induction, although perceived in the leaves, alters cytokinin metabolism in the stem and root tissues rather than in the leaves themselves.

Metabolism in Detached Leaves. In view of the apparent similarity in BA derivatives found in leaves from LD, SD, and SD + GA shoot systems, it was considered of interest to study metabolism of BA applied directly to detached leaves to avoid limitations posed by the transport system (Fig. 5, C and D). The petioles of mature and young, expanding leaves from SD-induced, SD + GA, and LD plants were immersed in [^{14}C]BA and 24 h later the blades were extracted and analyzed as usual. There was considerable metabolism of BA, but as with the intact shoots, very little difference between leaves from LD, SD, and SD + GA treated plants was detected. BA is converted to BAR and the polar derivatives. Relatively more of the polar derivatives appear in the young than in the mature leaves, but there is no apparent influence of GA or daylength on the relative amounts of the different metabolites of BA in either the young or mature leaves.

Metabolism in Apical Buds. [^{14}C]BA was applied directly to the shoot tip of plants that had received 6 h SD or remained in LD. After 12 or 24 h incubation the 1.5-cm-long apical bud was excised, rinsed, and extracted as before. In other experiments the basal 0.5 cm of excised apical buds, 3 cm long from which all unfolded leaves were removed, from LD and SD plants were immersed in [^{14}C]BA solutions for 24 h. The apical 1.5 cm of the buds were excised, extracted, and the extracts analyzed as before. Since there was no difference in metabolism between the intact and detached buds, only the data for the attached system are presented.

After 24-h incubation there was little apparent difference in BA metabolism between apical buds taken from LD or SD plants, only the scan for the SD plants is presented in Figure 6A. That is, in both cases nearly all of the recovered ^{14}C was found in the polar region. GA treatment of the SD plants produced a substantial change in metabolic pattern. Most of the ^{14}C recovered was in the BA zone, with a moderate sized peak in the polar region and a relatively minor peak in the BAR zone (Fig. 6B). There may have been differences in the rates of BA metabolism between apical buds from LD and SD plants that went undetected, perhaps due to the length of the incubation treatment. Thus, in another series of experiments, apical buds were incubated with [^{14}C]BA for 12 h. Analyses of these extracts revealed differences in metabolic patterns between the SD and LD buds (Fig. 6, C and D); as expected most of the activity extracted from the SD buds was in the polar region. Eluted radioactivity from the TLC plates (Table II) revealed a substantial fraction of BAR in the LD buds, 60-fold more than in the SD buds. This is the only indication that the riboside may be an important intermediate in BA metabolism in the apical tissues.

GA Influence on [^{14}C]BA Metabolism and Distribution in Shoot System. TLC analyses of extracts of detached apical buds incubated with [^{14}C]BA reveal that GA treatments to the apical bud 24 h before incubation cause a considerable reduction in the formation of the polar complex (Fig. 6). Without GA treatment both LD- and SD-induced apical tissues convert all of the absorbed [^{14}C]BA to the polar complex in a 24-h incubation period. Note that in the GA-treated tissues there is little BAR relative to either the polar complex or free base.

Spray applications of GA, 24 h before incubation with [^{14}C]BA, greatly affect BA distribution and metabolism in all tissues. Leafy

Table I. *Metabolism of BA*

A: distribution of ^{14}C from $[^{14}\text{C}]\text{BA}$ in shoot systems from LD- and SD-induced plants. B: in a separate experiment GA (1 mg/l) was spray-applied to SD-induced plants 24 h before incubation of the detached leafy shoot system in $[^{14}\text{C}]\text{BA}$. Comparisons are shown for SD-induced plants not treated with GA.

| Treatment | Total ^{14}C | | Stem | | Mature Leaves | | Young Leaves | | Apical Bud | |
|-----------|-----------------------|---------|---------|-------|---------------|-------|--------------|------|-------------|------|
| | dpm | dpm | dpm | % | dpm | % | dpm | % | dpm | % |
| A. SD | 191,832 | 149,696 | 149,696 | 78.03 | 28,116 | 14.65 | 13,595 | 7.08 | 425 | 0.22 |
| LD | 142,585 | 129,896 | 129,896 | 91.10 | 8,279 | 5.80 | 4,130 | 2.89 | 280 | 0.19 |
| Treatment | Total ^{14}C | | Stem | | Mature Leaves | | Young Leaves | | Apical Buds | |
| | dpm | dpm | dpm | % | dpm | % | dpm | % | dpm | % |
| B. SD | 399,435 | 343,920 | 343,920 | 86.10 | 49,430 | 12.37 | 5,625 | 1.40 | 460 | 0.11 |
| SD + GA | 272,832 | 254,140 | 254,140 | 93.14 | 16,680 | 6.11 | 1,552 | 0.56 | 460 | 0.16 |

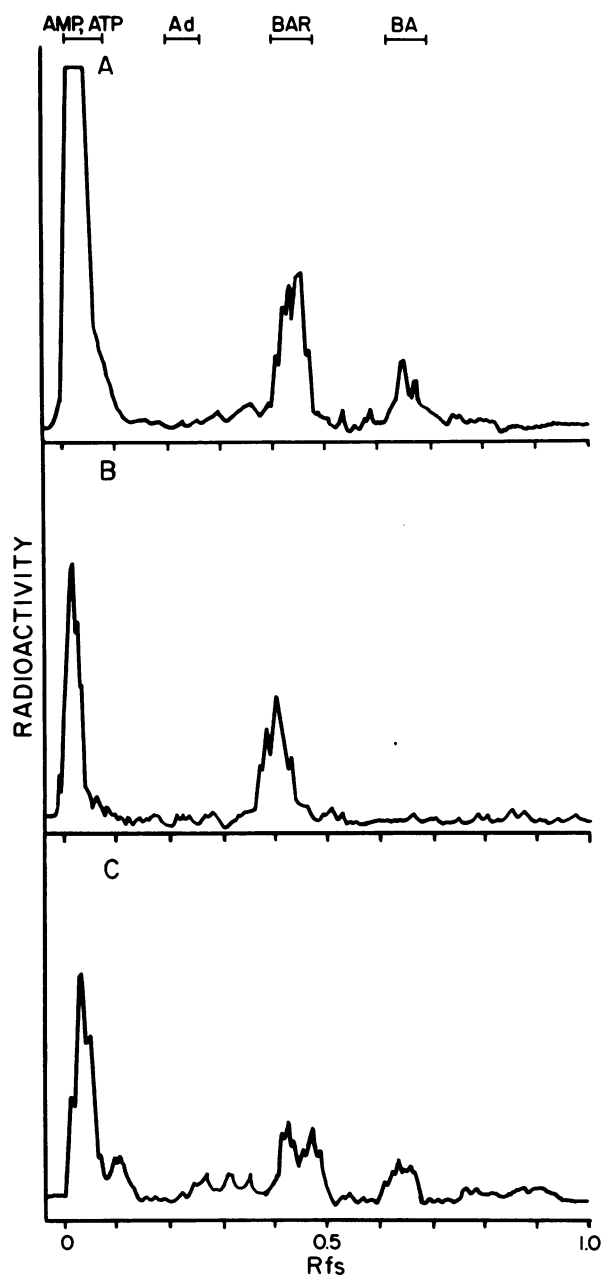


FIG. 4. Traces of ^{14}C scans of TLC. Extracts of stem tissues from SD (A), LD (B), and SD + GA (C) plants incubated with $[^{14}\text{C}]\text{BA}$ for 24 h. GA (1 mg/l), was spray-applied 24 h before incubation with $[^{14}\text{C}]\text{BA}$.

shoot systems, detached from their roots and immersed in $[^{14}\text{C}]\text{BA}$ for 24 h before ^{14}C analysis, show the influences of an earlier GA treatment on BA distribution (Table I). GA reduces the total

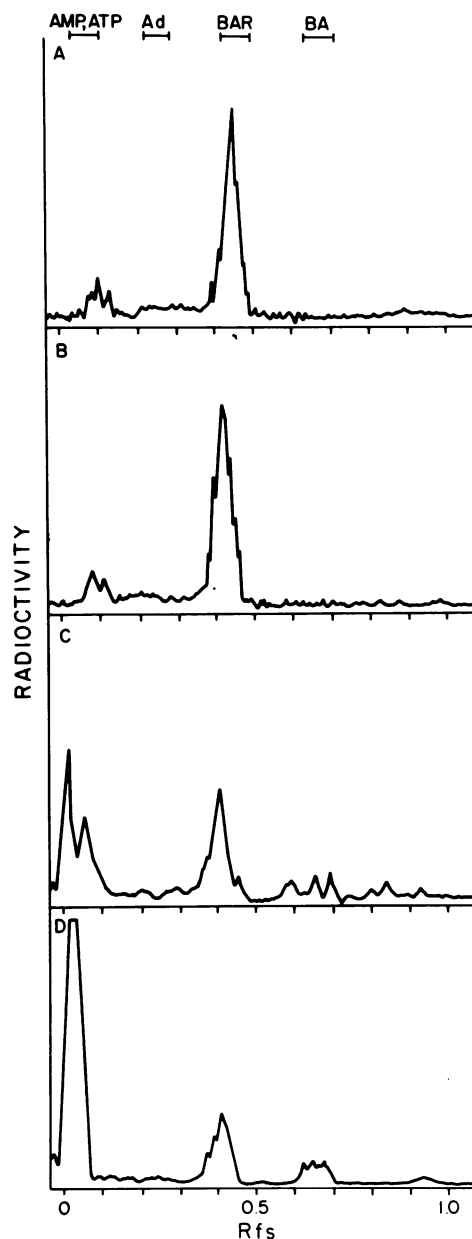


FIG. 5. Traces of ^{14}C scans of TLC. Extracts of mature (A) and young (B) leaf tissues from SD plants (as in Fig. 4); C (mature) and D (young) are scans of extracts for detached leaves from SD plants. Radiochromatograms for extracts of leaves from LD and SD + GA plants were nearly identical and are not shown.

^{14}C absorbed by the shoot, in particular the amount moving into the leaves. Chromatographic analyses of the extracts of the stem tissues reveal proportionately less of the polar derivatives in the

GA-treated than in the untreated plants (Fig. 4C). Very little free base is detected in either case, suggesting that in the stem tissues the only mobile forms of BA are the riboside or more polar derivatives, such as the ribotide.

DISCUSSION

These findings are significant because they show strong influences of day length and GA on BA metabolism and suggest hypotheses concerning interactions among hormonal and environmental parameters in the control of flowering in *Bougainvillea* (17). In both apical and root tissues BA is metabolized more rapidly to polar derivatives in SD than LD plants. GA applications to SD-induced plants reduce the rate of BA metabolism to that found in LD plants. The leaves are fully capable of forming the more polar complex when treated directly, and both LD- and SD-induced leaves metabolize BA in apparently the same fashion. That is, SD induction, regulating BA metabolism in the root, apical, and lower stem tissues, does not seem to alter metabolism in the leaves which are the major daylength sensitive tissues in *Bougainvillea*. Since the roots are the presumptive sites for cytokinin synthesis (6), it is reasonable that the SD signal would be sent to these tissues if the naturally occurring cytokinins are to play a major role in controlling flowering. Altered metabolism in the apical bud is expected if cytokinin is synthesized in the bud as well as the roots or if the translocatable form must be converted to another compound to promote inflorescence development.

The data presented for GA influences on BA metabolism support (more correctly, do not disprove) the hypothesis that SD induction may promote flowering in *Bougainvillea* by reducing gibberellin levels in certain critical tissues, thereby increasing the formation of BA ribotides and other adenyl derivatives which are either directly or indirectly active in promoting primordial initiation. There are severe limits that must be placed on this hypothesis since data are not yet available on day length influences on the metabolism or distribution of naturally occurring gibberellins or cytokinins in *Bougainvillea*. Studies in progress on the distribution of [^3H]GA₁ as a function of day length show that much less of that gibberellin is transported from young leaves in SD than in LD conditions. Results of earlier studies, showing that 2-chloroethyltrimethyl ammonium chloride, an apparent inhibitor of gibberellin biosynthesis in some species, promotes flowering in *Bougainvillea* (5), lend support to the hypothesis that reduced gibberellin levels are required for primordial initiation. Note that GA does not appear to alter cytokinin metabolism in the leaves but only in the root and apical bud tissues. We should like to emphasize that if SD induction in *Bougainvillea* is indeed a means of reducing gibberellin levels in the critical apical and root tissues, and if this is a part of the system controlling reproductive development, then one is concerned with a "negative" signal from the induced leaves; *i.e.* the reduction of a translocatable factor may constitute a part of the floral stimulus in *Bougainvillea*.

What is the significance of the finding that only the polar derivatives of BA are found in SD-induced plants in the critical apical tissues? BAR and BAR ribotide are reported to be storage forms of BA primarily because they are less active than the free base in bioassays (4, 12, 20). BA is readily incorporated as the ribotide into ribosomal and transfer RNA (2, 3). Such incorporation is not believed essential for its primary growth-promoting

role since a substituted BA, not incorporated into RNA, is as active as free BA in soybean callus tissue (7). Thus, one is left with the conclusion that BA itself or some other as yet unidentified derivative is the biologically active form in several assays. This conclusion, however, does not offer a satisfactory explanation for

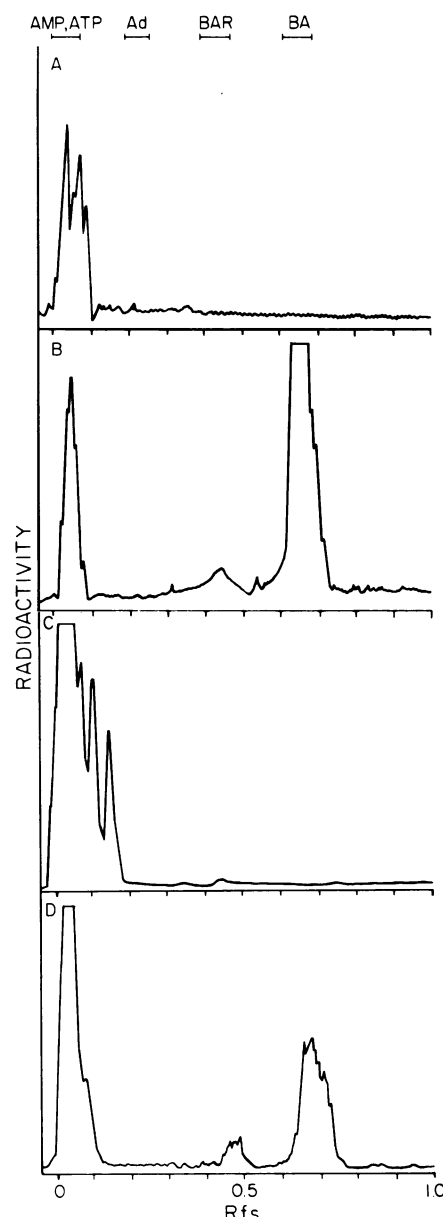


FIG. 6. Traces of ^{14}C scans of TLC. Extracts of apical buds from SD (A) and SD + GA (B) plants incubated for 24 h in [^{14}C]BA. Radiochromatograms for extracts from LD apical buds were nearly identical to SD and hence are not shown. GA (10- μl droplets of 1 mg/l) was applied to the apical bud by microsyringe 24 h before application of [^{14}C]BA. Traces C and D are scans of extracts of apical buds for SD (C) and LD (D) plants incubated with [^{14}C]BA for 12 h.

Table II. Metabolism of [^{14}C]BA in Apical Buds from SD-induced and LD Plants

Extractions made 12 h after the beginning of incubation; chromatographic analyses as before; scans as in Figure 6 (C and D); areas under peaks eluted and counted in a liquid scintillation counter.

| | Fresh Weight Buds mg | ^{14}C at Origin | | ^{14}C at BAR | | ^{14}C at BA | | Total ^{14}C dpm |
|----|-------------------------|---------------------------|---------|------------------------|---------|-----------------------|---------|------------------------------|
| | | dpm | % total | dpm | % total | dpm | % total | |
| SD | 175 | 608,640 | 99.64 | 1,520 | 0.24 | 640 | 0.07 | 610,800 |
| LD | 163 | 415,320 | 84.00 | 61,520 | 12.44 | 17,560 | 3.55 | 494,400 |

the dominance of the BA-related polar derivatives in apical buds of *Bougainvillea* nor would one have expected to find that SD induction decreased the amount of free BA to the advantage of the polar derivatives if the free base were the active form. It is possible that a heretofore unidentified polar derivative of BA, not a ribotide, perhaps not even a phosphorylated compound, is the active form.

Do these results with BA, GA, and SD serve as a guide to the interactions of naturally occurring cytokinins and gibberellins and day length in the control of reproductive development in *Bougainvillea*? It must be noted that neither zeatin nor kinetin promotes primordial initiation in *Bougainvillea* (19). Although it is possible that the inactivity of these cytokinins may be related to their failure to penetrate *Bougainvillea* tissues or to their rapid inactivation, one must consider the possibility that the action of BA in reproductive development is peculiar to its structure and not characteristic of all cytokinins (all of which are active in *in vitro* cell division assays). BA and kinetin have flower-promoting activities in several species (1, 8, 10, 13, 14, 17, 18), whereas published studies show that zeatin inhibits reproductive development where it is at all active (11). Thus, it is a moot question whether in *Bougainvillea* SD and GA will have the same effect on metabolism of naturally occurring cytokinins as they do on BA.

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