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Uptake and Metabolism of [^{14}C]Salicylic Acid in *Lemna gibba* G3[1,2]

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ABSTRACT

When the long-day plant *Lemna gibba* L., strain G3 is grown under continuous light on ammonium-free half-strength Hutner's medium (NH_4^+-free 0.5 H medium) there is virtually no flowering, but addition of 10 micromolar salicylic acid (SA) to the medium results in substantial flowering. Using this system, the uptake and metabolism of [^{14}C]SA in *L. gibba* G3 has been examined. SA uptake is rapid and linear for at least the first 24 hours. After 30 minutes, nearly 90% of the radioactivity in the plants is present as free SA. Part of this is rapidly converted to one or more bound forms of SA that appear either in the acidic butanol fraction or in the aqueous residue, and after 12 hours an equilibrium is reached between the free and bound forms of SA. When plants receive SA for 6 days and then are switched to control medium, both the free and bound SA remain nearly constant for at least 5 days. However, there is virtually no transfer of SA from mother fronds to daughter fronds, indicating that the SA is apparently sequestered within the cell. Cell fractionation studies show that nearly 95% of the SA remains in the supernatant even after a 2-hour centrifugation at 300,000*g*. Thus, it is unlikely that SA is confined within a specific organelle, but rather is probably secreted into the vacuole.

When *Lemna gibba* G3 is grown on E medium (Hoagland-type medium), it exhibits a qualitative long-day flowering response (3, 4). By contrast, on NH_4^+-free 0.5 H medium[4] there is virtually no flowering even under continuous light (10). Growth is excellent on both media, but the rate of new frond production is considerably higher on the NH_4^+-free 0.5 H medium. SA rapidly reverses both the inhibition of flowering and the stimulation of the growth rate due to NH_4^+-free 0.5 H medium (2,10). The optimal SA concentration for this effect is about 10 μM (10). Once SA is removed from the medium, its effect on both flowering and growth disappears very quickly, and thus it must be present in the medium for the entire experiment to give the optimal response (2).

These results raised the questions of why the SA effect disappears so quickly when it is removed from the medium, and what is the metabolic fate of SA once it is taken up by the plants. The present study addresses these questions by utilizing [^{14}C]SA to study the uptake and metabolism of SA in *L. gibba* G3 when grown on NH_4^+-free 0.5 H medium.

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[4] Abbreviations: NH_4^+-free 0.05 H medium, ammonium-free half-strength Hutner's medium; SA, salicylic acid.

MATERIALS AND METHODS

Plant Material. All work was done with aseptic cultures of the long-day plant *Lemna gibba* L., strain G3.

Culture Conditions. The maintenance of stock cultures, procedures for obtaining the plant material used to start experiments, and the temperature and light conditions used to grow the plants were the same as previously described (1). All cultures were grown in 125-ml Erlenmeyer flasks with 50 ml of medium. Experimental cultures were grown on NH_4^+-free 0.5 H medium that contained 1% sucrose and was prepared using deionized H_2O with a minimum resistance of 18 megohms. The medium was prepared at pH 6.2 and autoclaved for 18 min at 1.1 kg/cm^2. SA was usually added to the medium prior to autoclaving, since autoclaving does not cause significant losses of the biological activity of SA (4), nor of radioactivity from [^{14}C]SA (preliminary experiments). However, sterile filtration was used to add SA to the medium in experiments that were already in progress.

Experimental Procedure. Depending upon the experiment, flasks were inoculated with either one, three, or five 4-frond colonies (1). Preliminary studies indicated that for experiments of up to 8 d duration, the SA effect on flowering and growth was not significantly altered by using more than one 4-frond colony to inoculate a flask. Each experiment was repeated at least once and usually several times, but only the results of a single, representative experiment are presented. SA was always used at a concentration of 10 μM. [7-^{14}C]SA with a specific activity of 51.7 mCi/mmol was purchased from New England Nuclear. For preparation of radioactive media, the [^{14}C]SA was mixed with nonradioactive SA to yield a chemical concentration of 10 μM and a specific activity in the medium of 2 mCi/mmol.

When plants were switched from radioactive to control medium, they were rinsed twice with sterile deionized H_2O and once with sterile control medium before being transferred to the control medium. The level of radioactivity in the control medium rinse was always very low.

In one experiment, 160 4-frond colonies were selected from cultures grown for 6 d with [^{14}C]SA and transferred to control medium (8 colonies/flask). The four fronds in each colony were designated as A (oldest frond), A-1 (first daughter frond of A frond), A-2 (second daughter frond of A frond), or A-1-1 (first daughter frond of A-1 frond) (Fig. 1). The 4-frond colonies were allowed to grow on control medium for 6 d until each colony had grown into approximately 16 fronds. At that time, the A, A-1, A-2, and A-1-1 fronds were separated from the other fronds. Methanol extracts were prepared from both groups of fronds and the level of radioactivity determined without further purification.

Extraction Procedure. The plants were first rinsed several times with water to remove any medium radioactivity. They were then ground in a mortar and pestle with a small amount of acid-washed sand. This was followed by overnight extraction with cold 80% methanol. The mixture was filtered, the plant residue reextracted for an additional 2 h with more cold 80% methanol, and filtered

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second time. The filtrates were combined and are referred to as the methanol extract. Sufficient K-phosphate buffer, pH 8.2, was added to ensure that the aqueous solution left after evaporation of the methanol would be approximately 0.25 M phosphate. The pH of this solution was always about 8.0. The extraction procedure outlined in Figure 2 was used to fractionate the methanol extracts. For extraction of the combined culture medium from all replicate flasks, the volume was reduced to about 50 ml by rotary evaporation, phosphate buffer added to yield 0.25 M phosphate, and this buffer solution partitioned according to the procedure in Figure 2, except that the petroleum ether step was omitted. In some cases, the mild acid hydrolysis (2 h, pH 1, 60°C) proved to be insufficient, so a severe acid hydrolysis (24 h, 6 N HCl, 100°C) was used instead.

Chromatography. TLC was done on precoated silica gel G plastic plates. They were developed in chloroform:ethyl acetate:acetic acid (60:40:5, v/v/v) or in chloroform:ethyl acetate:methanol:acetic acid (60:40:10:5, v/v/v/v) for the acidic ethyl acetate fraction. The acidic butanol fraction was chromatographed in 1-butanol:methanol:water (80:15:15, v/v/v). Authentic SA was used as a marker in both cases. Plates were divided into 10 equal R_F zones. Each zone was cut out and inserted into scintillation vials for counting. In some cases, specific R_F zones from the TLC plate of the acidic butanol fraction were eluted with 80% methanol. The methanol was removed by rotary evaporation and the resulting aqueous solution subjected to severe acid hydrolysis. This was followed by partitioning against ethyl acetate at pH 2.5 and TLC of the acidic ethyl acetate fraction according to the procedure listed above.

Scintillation Counting. Radioactivity was determined with a LKB 1215 Rackbeta II liquid scintillation counter. Several replicate 1-ml samples of each fraction were added to the scintillation vials along with 9 ml of Ultrafluor (National Diagnostics). The TLC plates were cut into specific R_F zones and the pieces were introduced into scintillation vials along with 9 ml of Ultrafluor. A quenching curve was determined and introduced into the counter program to calculate dpm automatically from the actual cpm.

Cell Fractionation. Plants that had grown in radioactive medium for different periods of time were gently ground in a mortar and pestle in 0.5 M phosphate buffer, pH 7.5, which contained 0.4 M sucrose, 0.2% BSA, and 0.01 M $MgCl_2$. The extract was first centrifuged for 5 min at 1000*g* to remove all unbroken cells, cell debris, and the sand. The supernatant was then centrifuged at 5000*g* for 10 min to sediment predominantly chloroplasts, at 20,000*g* for 30 min to sediment predominantly mitochondria, at 125,000*g* for 30 min to sediment ribosomes and large membrane fragments, and at 300,000*g* for 2 h to sediment any remaining membrane fragments and large protein molecules. Each pellet was resuspended and they, as well as the 300,000*g* supernatant, were counted for radioactivity without further purification.

RESULTS

Uptake of SA and Distribution of Radioactivity in Different Fractions. Plants were grown for 11 d on medium containing [^{14}C]SA. They were extracted with methanol and the extract fractionated by solvent partitioning (Table I). The majority of the radioactivity was found to partition either into ethyl acetate at pH 2.5 (acidic ethyl acetate fraction) or into water-saturated 1-butanol at pH 3.0 (acidic butanol fraction). There was also considerable radioactivity left in the aqueous residue and only a relatively small portion of this would partition into ethyl acetate at pH 2.5 following a mild acid hydrolysis. Thus, in subsequent experiments, the mild acid hydrolysis was usually eliminated and attention was focused just on the acidic ethyl acetate fraction, the acidic butanol fraction, and the remaining aqueous residue.

Extracts were also prepared from the 11-d culture medium. Approximately 61% of the total radioactivity present in the medium at the start of the experiment was still present in the medium after 11 d. When the extracts of the medium were fractionated, about 76% of the radioactivity partitioned into the acidic ethyl acetate fraction (results not shown).

In short-term experiments, the uptake of [^{14}C]SA, as measured by the total radioactivity in the methanol extract, was linear for at least the first 24 h (Fig. 3). However, the distribution of the radioactivity between the different fractions showed marked changes. After 30 min, nearly 90% of the radioactivity was in the acidic ethyl acetate fraction, but the percentage dropped rapidly to around 30% at 12 h. By contrast, both the acidic butanol

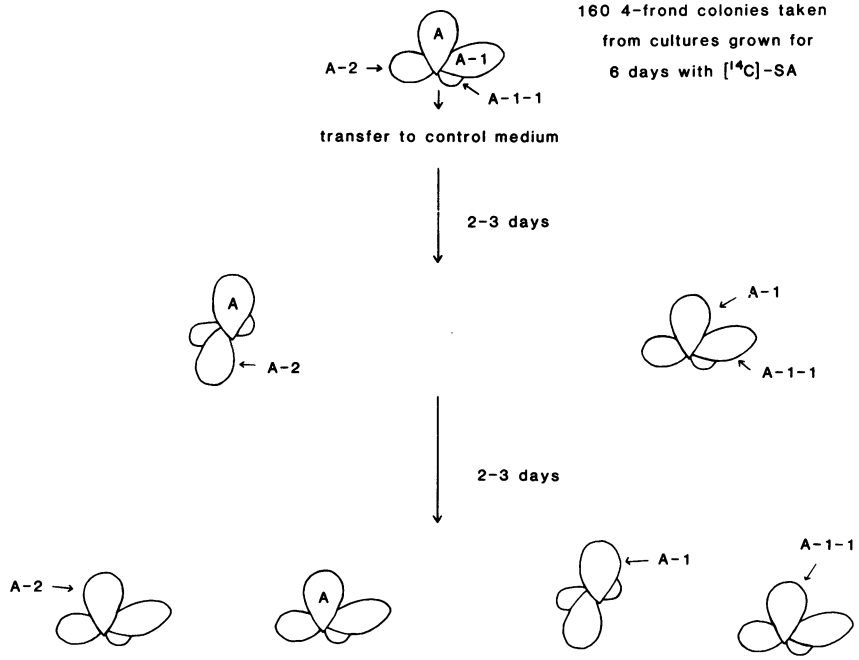

FIG. 1. Experimental procedure used to measure possible movement of [^{14}C]SA from mother fronds (A, A-1, A-2, A-1-1) to daughter fronds (all other fronds).

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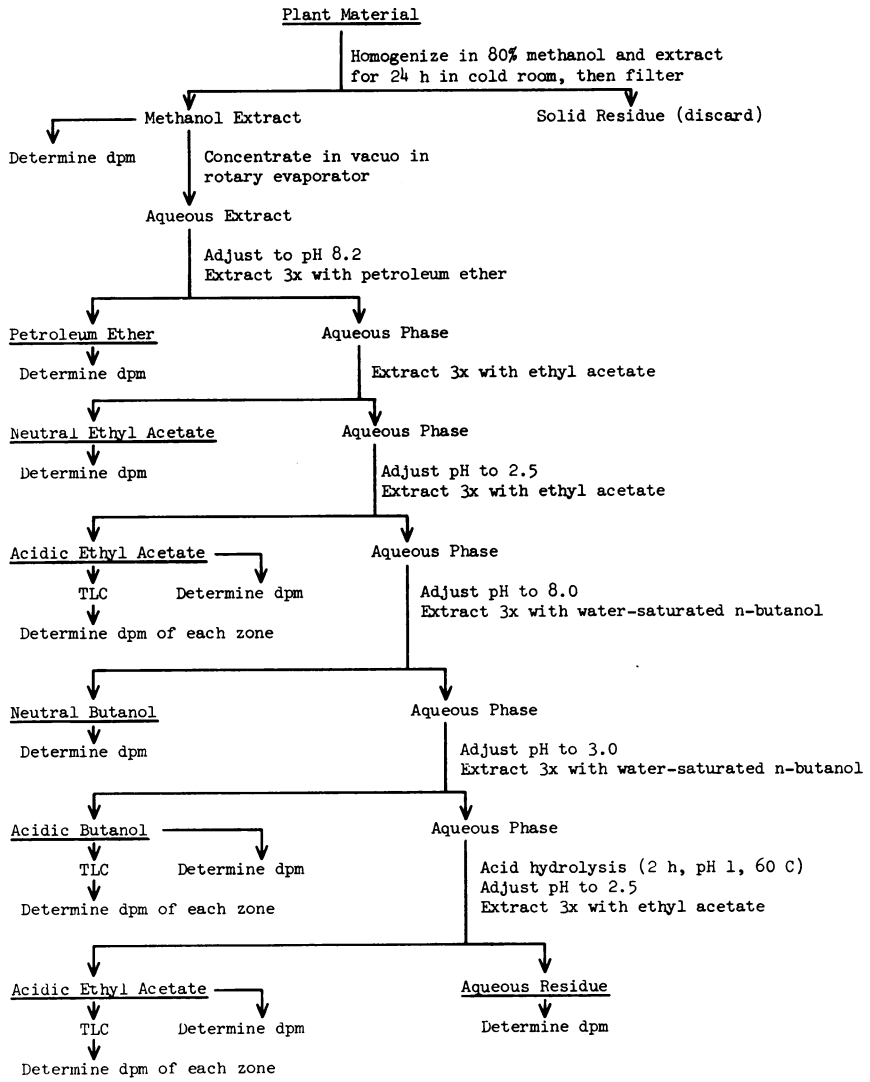

FIG. 2. Procedure for solvent fractionation of methanol extracts.

Table I. *Distribution of Radioactivity in Different Fractions following Solvent Partitioning of Methanolic Extracts of L. gibba G3*

Plants were grown for 11 d with 10 μM [^{14}C]SA. Acid hydrolysis treatment was 2 h, pH 1, 60°C.

Fraction	*dpm/flask*	%
Methanol extract	448,800	100.0
Petroleum ether, pH 8.2	8,800	2.0
Ethyl acetate, pH 8.2	4,300	1.0
Ethyl acetate, pH 2.5	118,500	26.4
Butanol, pH 8.0	27,400	6.1
Butanol, pH 3.0	167,800	37.4
Ethyl acetate, pH 2.5 (after mild acid hydrolysis)	17,700	3.9
Aqueous residue	58,400	13.0

fraction and the aqueous residue contained only about 5% of the radioactivity after 30 min, but the percentage in the aqueous residue quickly increased to about 20% after 3 h, and the percentage in the acidic butanol fraction also increased substantially and was nearly 50% at 12 h. Thus, there is a rapid change in the proportion of the radioactivity that partitions into each fraction, and after 12 to 24 h an equilibrium is established that persists at least through 11 d.

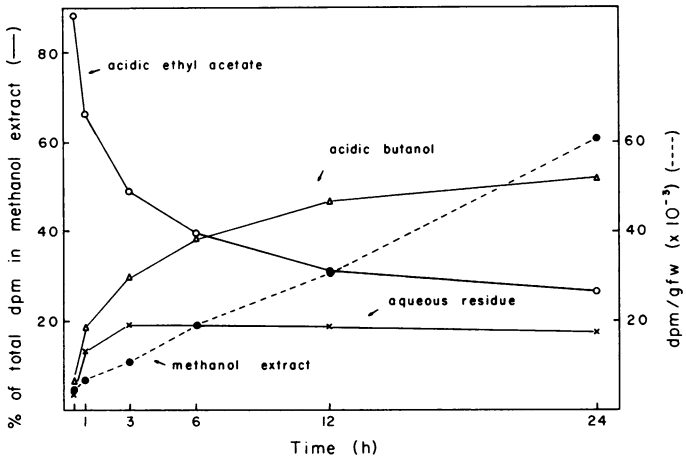

FIG. 3. Time course for uptake of [^{14}C]SA and its distribution in different fractions. Plants were grown for 5 d on control medium, and then [^{14}C]SA was added by sterile filtration and uptake was allowed to proceed for indicated time periods.

The effect of SA on flowering and growth ceases almost as soon as SA is removed from the medium (2). Therefore, it was of interest to determine the fate of SA left within the plants after SA

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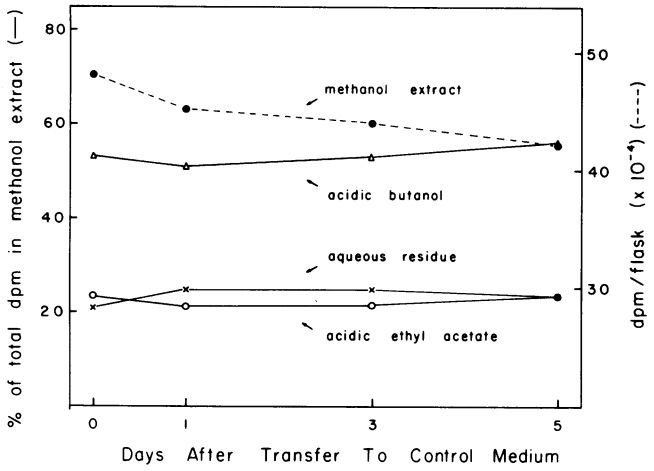

FIG. 4. Distribution of radioactivity in different fractions from plants exposed to [^{14}C]SA for 6 d and then switched to control medium for indicated time periods.

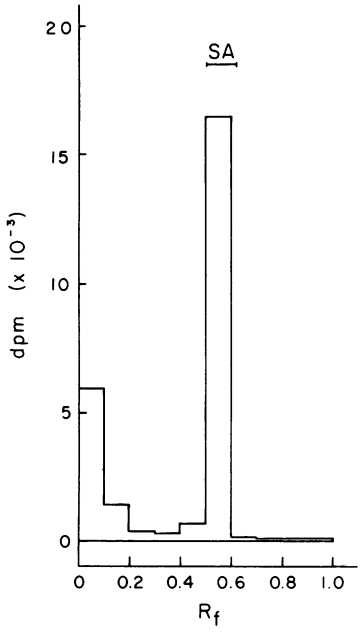

FIG. 5. Distribution of radioactivity in acidic ethyl acetate fraction from plants treated for 24 h with [^{14}C]SA.

was removed from the medium. To do this, plants were grown for 6 d on medium containing [^{14}C]SA and then switched to control medium for an additional 1, 3, or 5 d (Fig. 4). Fractionation of the methanol extracts showed that the percentage of the radioactivity that was found in the acidic ethyl acetate fraction, acidic butanol fraction, and the aqueous residue remained practically constant throughout the experiment. A very small amount of radioactivity appeared in the culture medium, but after 5 d it was less than 5% of that contained in the plant extracts. The amount of radioactivity in the methanol extracts of the plants showed only a slight decline when it was expressed on a per flask basis. However, when expressed in terms of fresh weight there was a 77% decline in radioactivity by day 5 because of continued growth of the plants in the absence of any additional uptake of [^{14}C]SA.

Chromatography. Plants treated with [^{14}C]SA for 24 h were extracted and the acidic ethyl acetate fraction subjected to TLC (Fig. 5). The major zone of radioactivity cochromatographed very closely to authentic SA and showed similar fluorescence. There was often an additional, much smaller zone of radioactivity at the origin, but no attempt was made to identify it. Similar results were obtained with extracts of culture medium, and it is thus assumed that the majority of the radioactivity in the acidic ethyl acetate fraction was present as free SA.

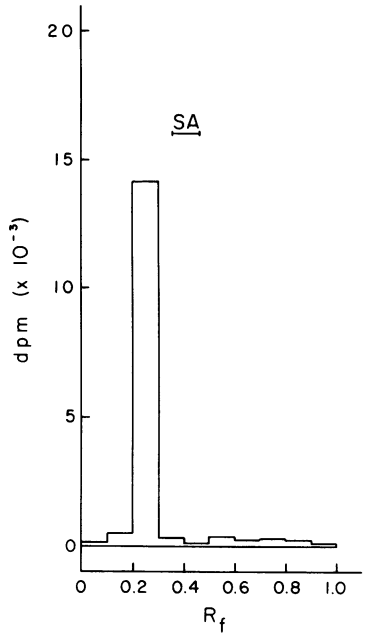

FIG. 6. Distribution of radioactivity in acidic butanol fraction from plants treated for 6 d with [^{14}C]SA.

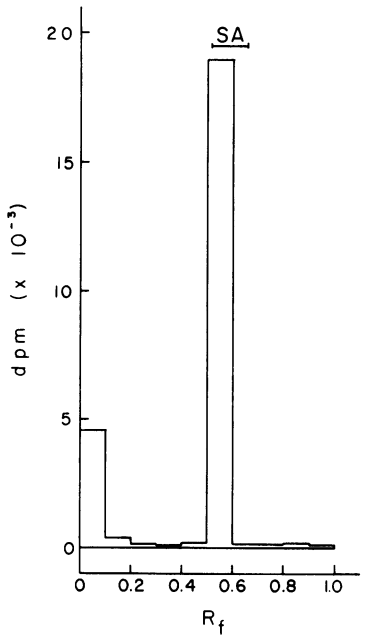

FIG. 7. Distribution of radioactivity in acidic ethyl acetate fraction following severe acid hydrolysis of acidic butanol fraction from plants treated for 6 d with [^{14}C]SA. The acid hydrolysis treatment was 24 h, 6 N HCl, 100°C.

In another experiment, plants treated with [^{14}C]SA for 6 d were extracted and the acidic butanol fraction subjected to TLC (Fig. 6). A single zone of radioactivity was found that showed similar fluorescence to SA but did not cochromatograph with authentic SA. On the assumption that this band might correspond to a bound form of SA, it was eluted and subjected to mild acid hydrolysis. However, very little free SA was obtained. Instead, a severe acid hydrolysis was tried, and following this treatment

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Table II. *Level of Radioactivity in Different Subcellular Fractions from L. gibba G3 Exposed to [^{14}C]SA for 1, 6, or 24 Hours*

Plants were extracted in 0.05 M phosphate buffer, pH 7.5, that contained 0.4 M sucrose, 0.2% BSA, and 0.01 M $MgCl_2$.

Fraction	1 h		6 h		24 h	
	dpm	%	*dpm*	%	*dpm*	%
1,000*g*, 5 min	480	1.8	910	1.8	3,100	1.8
5,000*g*, 10 min	160	0.6	740	1.5	3,270	1.9
20,000*g*, 30 min	30	0.1	120	0.3	800	0.5
124,000*g*, 30 min	100	0.4	120	0.3	780	0.5
300,000*g*, 2 h	50	0.2	170	0.4	520	0.3
Supernatant from 300,000*g* fraction	25,800	96.9	48,000	95.7	169,200	95.0

Table III. *Level of Radioactivity in Mother and Daughter Fronds*

See text for experimental procedure used to obtain the two groups of fronds. Plants were grown for 6 d with [^{14}C]SA. Methanol extracts were prepared from each group of fronds and counted for radioactivity without further purification.

Extract	Fresh wt		
	g	*dpm/g fresh wt*	%
Mother fronds (A, A-1, A-2, A-1-1)	8.40	84,500	95.5
Daughter fronds (all other fronds)	13.10	4,000	4.5

about 94% of the radioactivity that eluted from the radioactive band seen in Figure 6 partitioned into the acidic ethyl acetate fraction, and TLC showed that the vast majority of this radioactivity cochromatographed almost exactly with authentic SA (Fig. 7).

When the aqueous residue was subjected to severe acid hydrolysis and then partitioned against ethyl acetate at pH 2.5, about 85% of the radioactivity originally present in the aqueous residue partitioned into the acidic ethyl acetate fraction. TLC of this fraction showed that most of the radioactivity cochromatographed with authentic SA. Thus, it seems clear that most of the radioactivity that is found in the acidic butanol fraction and the aqueous residue is present in one or more bound forms of SA.

Cell Fractionation. An effort was made to determine whether SA might be associated with a particular cell organelle in plants exposed to [^{14}C]SA for 1, 6, or 24 h (Table II). With longer exposures, the level of radioactivity increased, but the percentage found in the different fractions remained virtually constant, and in each case about 95% of the radioactivity remained in the supernatant even after a 2 h centrifugation at 300,000*g*. These results strongly indicate that the SA is not associated with a particular cell organelle, but is localized in the cytoplasm and/or in the vacuoles.

Examination for Transfer of SA from Mother Fronds to Daughter Fronds. One hundred and sixty 4-frond colonies were selected from cultures grown for 6 d with [^{14}C]SA, transferred to control medium, and allowed to grow for 6 d until each colony had grown into 16 fronds (Fig. 1). The level of radioactivity in the four original mother fronds in each group of 16 (A, A-1, A-2, A-1-1), which had been directly exposed to [^{14}C]SA, was compared to that found in all of the daughter fronds. Since some culture medium penetrates into the reproductive pockets of a mature frond, a few of the larger daughter fronds probably received a slight exposure to [^{14}C]SA from the medium.

Nevertheless, over 95% of the radioactivity was present in the original mother fronds (Table III). In a duplicate experiment, 94% of the radioactivity was recovered from the original mother fronds. In a preliminary experiment where [^{3}H]GA_1 was used instead of [^{14}C]SA, only about 50% of the radioactivity was found in the original mother fronds with the rest recovered from the remaining daughter fronds, Thus, it seems clear that very little movement of SA from mother to daughter fronds must take place.

DISCUSSION

Extraction of plants growing on medium containing [^{14}C]SA shows that SA uptake is rapid and linear for at least the first 24 h. Initially, most of the radioactivity partitions into the acidic ethyl acetate fraction and cochromatographs with authentic SA, and thus, presumably is free SA. However, with time, part of this free SA is converted to one or more substances that partition into acidic butanol or stay in the aqueous residue. The studies utilizing severe acid hydrolysis indicate that most of the radioactivity that partitions into the acidic butanol fraction or remains in the aqueous residue consists of one or more bound forms of SA. One likely possibility is SA-glycosides, but no identification has been made.

The cell fractionation studies indicate strongly that the free and bound SA are not preferentially associated with a particular cell organelle or membrane system, and they suggest that SA is probably present either in the cytoplasm or in the vacuoles. If the SA was present in the cytoplasm, it would be expected to move via plasmadesmata to the daughter fronds. However, the results in Table III make it clear that there is virtually no transfer of SA from mother fronds to daughter fronds. It seems more likely that the SA is in the vacuoles. SA is somewhat toxic to the plants and it is well known that such substances are often rendered nontoxic by being moved into the vacuoles (7, 9). However, at present, there is no direct evidence to support this possibility.

Why does the SA effect on flowering and growth cease very quickly after SA is removed from the medium? One might postulate that this was due to a very rapid breakdown of SA once it was taken up by the plants. However, the results in Figures 3 and 4 clearly indicate that, quite to the contrary, both free and bound SA are quite stable and the equilibrium between them remains nearly constant even up to 5 d after SA is removed from the medium. A more likely possibility is that SA becomes inactivated very soon after being taken up, and if the medium does not contain more SA for the plants to take up the effect will quickly cease. The available evidence suggests that both the free and bound SA are quickly secreted into the vacuole, and if this is true it would presumably result in the inactivation of the SA.

If SA becomes inactive soon after uptake, it suggests that it may be acting at the level of the cell membrane during the process of being taken up or very soon thereafter. SA is known to cause rapid changes in ion permeability of roots (5), depolarization of root cells (6), and stomatal closure in *Phaseolus* (8). Studies in progress in this laboratory have shown that the inhibition of flowering in *L. gibba* G3 in NH_4^+-free 0.5 H medium can be partially reversed by increasing the phosphate concentration in the medium 10-fold. Perhaps SA is acting by influencing phosphate uptake or phosphate availability within the cell, or by influencing the uptake, secretion or intracellular distribution of some other iron or organic compound that has an influence on flowering.

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