total amount of drug dissoluted in a 22-hr. period.

6. In alkaline pancreatin solution, white wax matrices had the fastest rates of release, the largest particles had the slowest rates, and sorbitan monooleate consistently promoted faster release rates.

7. Good agreement was obtained between the model predictions and experimentally determined amounts of drug released from a formulation in a particular length of time. Determination of the model parameters adequately characterized the dissolution curve over the time period studied.

8. Sorbitan monooleate, when present in concentrations of 10%, was found to repress the dissolution rate of SETD in 0.1 *N* HCl, and to augment the rate in alkaline pancreatin solution. This behavior could be of use to formulators wishing to insure efficient release of the drug in the intestines after an initial delay.

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Keyphrases

Sulfaethylthiadiazole—wax formulations
Spray-congealed particles
Apparatus, particle preparation—described
Nozzle orifice size—dissolution rate
Sorbitan monooleate—dissolution rate
Waxes—dissolution rate

Effect of Gibberellic Acid on the Growth, Alkaloid Production, and VLB Content of *Catharanthus roseus*

By A. N. MASOUD, L. A. SCIUCHETTI, N. R. FARNSWORTH, R. N. BLOMSTER, and W. A. MEER*

***Catharanthus roseus*, treated for 7 weeks with weekly doses of 100 mcg. of gibberellic acid (GA) and harvested biweekly, showed a significant increase in height and in stem dry weight. While GA treatment had a favorable effect on growth and did not affect the rate of flowering, it had an unfavorable effect on the concentration of total alkaloids in the plant organs. At final harvest the concentration of total alkaloids in the leaves was 55 percent of controls; that of the roots, 39 percent. Total alkaloid content per plant was greater than controls at the second harvest, but decreased at the fourth harvest. Detectable quantities of vincaleukoblastine (VLB) were not found in the stems or roots of both treated and untreated groups, but an increase of VLB concentration was noted in the leaves of the treated group. Gibberellic acid treatment had no affect on chlorophyll content, but markedly reduced the petroleum ether and ether extracts of plant organs.**

THE FOLLOWING GENERAL gibberellin effects have been reported in the solanaceous plants producing tropane alkaloids: increased internodal elongation, taller and spindlier plants, increased stem growth, variable effects on total plant growth depending upon the stage of the plant when treated and the concentration of growth hormone applied as well as environmental factors, chlorotic leaves and reduced chlorophyll content, an increased sugar content of the leaf tops, and a reduction in the concentration of alkaloids of the aerial parts (1–6). Many of these effects have been reported for other alkaloid pro-

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ducing plants (1). Markiewicz (7, 8) reported that gibberellin treatment of *Catharanthus roseus* caused an increase in height but reductions in the weight of the aerial parts and the yield of root alkaloids.

The objectives of this research were: (*a*) to determine the effects of gibberellic acid (GA) on the growth of *Catharanthus roseus* (L.) G. Don (*Vinca rosea* L.) when the plant was grown under controlled greenhouse conditions during a 7-week observation period at which time periodic harvests were made; (*b*) to further ascertain the effects of GA on the concentration of alkaloids in the plant organs and the total alkaloid content of the plant; (*c*) to determine the effects of such treatments on the production of the antineoplastic alkaloid, vincaleukoblastine (VLB), during various growth phases of the plant; (*d*) to ascertain the effects of the growth regulator on other metabolic products.

EXPERIMENTAL

Procedure—*Catharanthus roseus* plants employed in this study were grown under greenhouse conditions from seeds obtained from a 1959 crop of mixed pink flowering and white flowering *C. roseus* (L.) G. Don grown in India from stock seed stored at the Department of Pharmacognosy, University of Pittsburgh. Germination was accomplished in flats containing a soil mixture of two parts of loam and one part of sand. About 50 Gm. of complete organic fertilizer[1] was incorporated into the soil of each flat. On July 24, 1964, the larger of the 10-day-old seedlings were transplanted from the flats into paper bands filled with the previously described soil mixture. These paper bands were arranged in flats, 80 per flat and a total of 12 flats were transplanted. One month later the plants were transplanted from the paper bands into individual 1-gal. metal containers that had been filled with the sandy loam mixture. Prior to transplantation approximately 15 Gm. of complete fertilizer had been thoroughly mixed into the soil of each container.

Plants of the pink-flowering variety, recognized by their pink stem were divided into two groups of 120 each. One group, numbered C-1 to C-120, served as the controls; the other, numbered GA-1 to GA-120, was the group treated with GA. These plants, numbered 111 through 120 for each group, were designated for height measurements and each group was further divided into subgroups of 30 plants each so that separate harvests at 2-week intervals could be performed. Plants were labeled according to the aforementioned treatment plan and randomized on two greenhouse benches.

Treatment was instituted on September 11 (zero time). At this time 0.1 ml. of a freshly prepared aqueous solution containing 100 mcg. of GA[2] was applied with a micropipet to the upper leaves of each plant of the GA group. Height measurements were also taken on the same date of the randomly arranged plants numbered 111–120 since these plants would be survivors throughout the whole experimental period. Height, in cm., was taken from the base of the stem at soil level to the apex of the highest leaf. Treatment and height measurements were performed at weekly intervals for 6 subsequent weeks. The 100-mcg. dosage was determined from a preliminary experiment employing dosage levels of 25, 50, and 100 mcg. of GA per group of three plants.

Four harvests were made at 2-week intervals. The first harvest was performed 5 days after the first treatment. Thirty plants were selected per harvest according to the following numbering plan: 1–30, first harvest plants; 31–60, second harvest plants; 61–90, third harvest plants; 91–120, fourth harvest plants. At harvest time each plant was thoroughly washed and excess moisture was removed with a towel. Plants were then divided into leaf, stem, and root portions; fresh weights were taken immediately, the respective plant parts were then dried in a circulating hot-air drier until dry weights were determined. The plant parts were pooled according to harvest and treatment, powdered to a No. 40 powder in a Wiley mill, and stored in air-tight glass containers until used for subsequent analysis.

Alkaloid Analysis—Samples of the pooled powdered material which had previously been dried in an oven at 100° for 24 hr. were assayed for total alkaloids in a manner similar to that of Szasz *et al.* (9). Five grams of the powdered crude drug was thoroughly moistened with 5 ml. of dilute ammonia soln. 58% (w/v), allowed to air dry and transferred, by washing with 75 ml. of chloroform, to a conical flask, stoppered, and allowed to macerate for 24 hr. This material was filtered through a Buchner funnel and the filtrate shaken with 100-ml. portions of 2% (w/v) sulfuric acid until it tested negative for alkaloids with modified Dragendorff's spray reagent. This acid solution was adjusted to pH 10 with dilute ammonia soln. 58% (w/v), and exhaustively extracted with chloroform. The resulting chloroform solution was dried over anhydrous sodium sulfate, filtered and concentrated to dryness, *in vacuo*, utilizing a methanol azotrope to assure complete removal of moisture. This residue was redissolved in chloroform, and adjusted to a volume of 25 ml. Three drops of indicator (0.5% w/v) solution of gentian violet in glacial acetic acid) and 12.5 ml. of glacial acetic acid were added, the mixture continuously stirred, and titrated to a visual green end point with 0.01 *N* perchloric acid in glacial acetic acid. Precautions were observed to assure that atmospheric moisture was not absorbed by the glacial acetic acid prior to its use in the titration procedure. The total alkaloid content was expressed as milligrams of perivine per gram of sample since this alkaloid is representative of the average molecular weight and structural type of alkaloids present in *C. roseus*. It is also the major alkaloid formed in the leaves of the plant (10).

The VLB content was determined from 1-Gm. samples of the pooled powdered plant material by a method devised in our laboratories and described in a recent publication (11).

[1] Organic Morcrop, Chas. Lilly Co., Seattle, Wash. (Analysis: 5% total nitrogen, 3% available phosphate, 2% available potash).

[2] Furnished through the courtesy of Dr. Edwin Alder, Agricultural Research Center, Eli Lilly and Co., Greenfield, Indiana.

Chlorophyll Content—The concentration of chlorophyll *a* and *b* was determined on 1/2-Gm. samples of powdered leaf material by a method previously described (4).

Selective Solvent Extraction—To determine the effects of the treatments on the formation of other types of metabolic products, duplicate 2-Gm. samples of the powdered dried leaves were selectively extracted in a Soxhlet apparatus in sequence with the following solvents: petroleum ether USP, absolute ether A.R., absolute alcohol A.R., and distilled water in a manner previously described (3).

RESULTS

A significant increase in height was induced by the GA treatment (Table I), and the maximal increase (61%) over controls occurred at the end of the second week. Just prior to the terminal harvest the GA-treated plants were 48% taller than controls. Analysis of variance of the height data (Table I) indicated that the height increases of the treated plants were statistically significant at the 1% confidence level. The rate of flowering was not affected by the gibberellin treatment.

A general favorable effect on plant growth was induced as indicated by dry weight data (Table II). Increases of 34, 20, and 14% were found in the total dry weight at the second, third, and fourth harvests, respectively. Stem dry weights were increased 11, 85, 93, and 40% over the controls at the first, second, third, and fourth harvests, respectively. The dry weight of the roots was increased except at the first harvest, but the weight of the leaves was not appreciably affected. Statistical analysis of the dry weight data indicated that there was significant increase in only the stem dry weights of the GA group. This increase was significant at the 5% level in only the second and third harvest plants. The marked increases in root and whole plant dry weights were not statistically significant.

First harvest samples were not assayed for the concentration of total alkaloids because insufficient amounts were available. Alkaloid concentration in the leaves of the controls increased with the age of the plant (Table III) and a reverse trend occurred in the GA group. In general, gibberellin treatment caused a reduction in leaf alkaloids, with the largest (45%) reduction occurring at the fourth harvest. Treated plants showed a lower alkaloid concentration in the stems than controls. In this case, both the treated and untreated groups demonstrated decreased alkaloid concentrations with age. Slight reductions were noted in the concentration of root alkaloids at the second and third harvest, and again the largest decrease (39%) occurred at the fourth harvest. It was observed that GA treatment generally induced a decrease in the concentration of alkaloids in all plant parts. This decrease was more pronounced in physiologically mature plants than in young plants. Alkaloid concentrations tended to increase with maturation in both leaves and roots, then leveled off at the final harvest.

Total alkaloid content in the leaves of treated plants was increased 31% at the second harvest but decreases of 20 and 45% were found at the third and fourth harvests (Table IV). This 31% gain was due both to increased growth and increased alkaloid concentration, while the reductions were due entirely to decreased alkaloid concentration. Total alkaloid content of the stems was increased at all three harvests (Table IV) and the increases were more pronounced at the early growth stages than during the more mature phases. These increases were due primarily to the effect GA had on increasing stem growth. Variable effects were noted on the total alkaloid content of the roots. The 32% decrease at the fourth harvest was due mainly to a significant reduction in the concentration of root alkaloids. The total alkaloid content per plant was increased 35% at the second harvest but decreased 39% at the final harvest, this latter effect was due

TABLE I—HEIGHT DATA OF PLANTS[a] DURING THE EXPERIMENTAL PERIOD

Measurement No. and Date	Controls, cm.	GA-Group, cm.	Control, %	L.S.D.[b] 0.05	L.S.D.[b] 0.01
1, Sept. 11[c]	8.90	9.55	107	...	...
2, Sept. 18	14.25	19.90	140	3.93	5.38
3, Sept. 25	20.20	32.55	161	5.82	7.98
4, Oct. 2	26.55	41.35	156	6.93	9.50
5, Oct. 9	31.25	47.60	152	7.53	10.32
6, Oct. 16	35.75	54.20	152	10.66	14.62
7, Oct. 23	38.65	57.25	148	10.96	15.01

[a] Average per group of 10 plants. [b] The lowest significant difference at the 5% and 1% confidence level calculated from the analysis of variance. [c] This measurement was performed prior to treatment on the date of the first treatment, 5 days prior to the first harvest.

TABLE II—WEIGHTS OF *C. roseus* PLANT PARTS[a]

Treatment and Harvest No.	Total Wt. Fresh, Gm.	Total Wt. Dry, Gm.	Total Wt. Control, Dry Wt., %	Leaves Fresh, Gm.	Leaves Dry Gm.	Leaves Control, Dry Wt., %	Stems Fresh, Gm.	Stems Dry, Gm.	Stems Control, Dry Wt., %	Roots Fresh, Gm.	Roots Dry, Gm.	Roots Control, Dry Wt., %
Control, 1	2.29	0.249	...	1.36	0.166	...	0.34	0.038	...	0.59	0.045	...
GA, 1	2.36	0.251	101	1.36	0.164	97	0.40	0.042	111	0.61	0.045	100
Control, 2	7.71	0.888	...	4.36	0.544	...	1.39	0.187	...	1.95	0.157	...
GA, 2	10.00	1.188	134	4.93	0.627	115	2.51	0.346	185	2.56	0.215	137
Control, 3	17.88	2.298	...	10.01	1.345	...	4.34	0.580	...	3.54	0.373	...
GA, 3	17.98	2.766	120	8.57	1.256	93	6.15	1.122	193	3.26	0.388	104
Control, 4	43.11	6.081	...	24.40	3.171	...	11.42	2.041	...	7.30	0.869	...
GA, 4	42.84	6.951	114	21.90	3.143	99	13.61	2.847	140	7.33	0.961	111

[a] Mean weights per group of 10 plants.

TABLE III—CONCENTRATION OF ALKALOIDS[a] OF *C. roseus* PLANT PARTS[b]

Treatment and Harvest No.	Leaves		Stems		Roots	
	Alkaloids, mg./Gm.	Control, %	Alkaloids, mg./Gm.	Control, %	Alkaloids, mg./Gm.	Control, %
Control, 2	5.3	...	4.2	...	7.7	...
GA, 2	6.1	115	4.1	98	6.7	87
Control, 3	7.0	...	2.9	...	8.7	...
GA, 3	6.0	86	2.4	83	7.9	91
Control, 4	7.3	...	2.0	...	8.6	...
GA, 4	4.0	55	1.7	85	5.3	61

[a] Expressed as perivine. [b] Determined from pooled samples of 30 plants per group.

TABLE IV—TOTAL ALKALOID CONTENT[a] (mg.) OF *C. Roseus*

Treatment and Harvest No.	Per Plant		Leaves		Stems		Roots	
	Total Alkaloids	Control, %	Total Alkaloids	Control, %	Total Alkaloids	Control, %	Total Alkaloids	Control, %
Control, 2	4.9	...	2.9	...	0.8	...	1.2	...
GA, 2	6.6	135	3.8	131	1.4	175	1.4	117
Control, 3	14.3	...	9.4	...	1.7	...	3.2	...
GA, 3	13.3	93	7.5	80	2.7	152	3.1	97
Control, 4	34.7	...	23.1	...	4.1	...	7.5	...
GA, 4	22.5	61	12.6	55	4.8	117	5.1	68

[a] Calculated from dry weight and alkaloid analyses data; per plant = leaves + stems + roots.

TABLE V—CONCENTRATION OF VLB[a] AND TOTAL CONTENT[b] OF LEAVES

Treatment and Harvest No.	VLB Concn.		Total VLB Content	
	mcg./Gm.	Control, %	mcg.	Control, %
Control, 1	30	...	5.0	...
GA, 1	36	120	5.9	118
Control, 2	28	...	15.2	...
GA, 2	31	111	19.4	128
Control, 3	30	...	40.4	...
GA, 3	28	93	35.2	87
Control, 4	19	...	60.2	...
GA, 4	24	126	75.4	125

[a] VLB = vincaleukoblastine. [b] Calculated from dry weight and VLB concentration data.

entirely to the appreciable reductions in the concentrations of alkaloids found in the plant organs.

One-gram samples of the dried plant organs were analyzed for vincaleukoblastine (VLB) by the previously described procedure. The concentration of VLB was less than the minimum detectable concentration of 5 mcg./Gm. in both the stems and roots. It was concluded that in both the treated and control groups grown under the aforementioned greenhouse conditions, these organs either contained no VLB, or, if the alkaloid was present, it occurred in concentrations below the detectable concentration of 5 mcg./Gm. dry weight of plant material. Concentration of VLB generally was higher in the leaves of the treated plants than controls (Table V). In the treated plants the concentration of VLB decreased in the leaves as the plants matured. Even in the controls, the leaf concentration was much higher at the first harvest than the fourth harvest. Since gibberellin treatment did not significantly affect the dry weight of the leaves, the total VLB content of the leaves followed the pattern shown for the VLB concentration.

Due to the small quantities of plant material remaining, only the leaves of the fourth harvest plants were assayed for the concentration of chlorophyll *a* and *b*. The concentration of chlorophyll *a* in the controls was 3.76 mg./Gm. compared with 3.94 mg./Gm. in the treated group. Corresponding figures for chlorophyll *b* were 3.45 and 3.57. The GA treatment had no significant effect on chlorophyll concentration.

Selective solvent extraction was performed on the various organs of the final harvest only (Table VI) and the petroleum ether and absolute ether extracts of all organs were markedly less in the treated group. The absolute ether extract of the stems of the treated group was about one-third of the controls. A slight increase over controls was noted in the alcoholic extract obtained from the leaves of the GA group whereas marked reductions were found in the stems and roots. No significant differences were noted in the aqueous extracts of the organs between treated and nontreated groups.

DISCUSSION AND CONCLUSIONS

A typical gibberellin effect characterized by increased internodal elongation which resulted in taller plants was noted in the treated plants. Analysis of variance of the height data indicated that the increased height of the GA group was

TABLE VI—RESULTS[a] OF SELECTIVE SOLVENT EXTRACTION OF FOURTH HARVEST ORGANS

Treatment	Plant Part	Petroleum Ether	Absolute Ether	Absolute Alcohol	Distilled Water
Control	Leaves	176	23	111	322
Control	Stems	131	30	95	193
Control	Roots	145	15	101	147
GA	Leaves	162	15	124	316
GA	Stems	110	11	60	173
GA	Roots	110	14	86	154

[a] Expressed as mg./Gm. dry wt.

significant at the 1% confidence level. Treatment with GA did not affect the rate of flowering, but growth of the plants, indicated by total dry weight, was favorably affected. As could be expected most of the weight gain was concentrated in the stems. The pattern for stem dry weight (over controls) of 85 and 93% at the second and third harvests, respectively, were statistically significant at the 5% confidence level. In general the dry weight of the leaves was not materially altered while slight gains were noted in root dry weights.

There was a general unfavorable effect of the GA treatment on the concentration of alkaloids in the various plant organs. This is in agreement with other investigations (1–6). Several trends were noted during the experimental period. The concentration of alkaloids in the leaves of the control group increased with the age of the plant while the reverse trend was noted in the GA group. Both treated and untreated groups demonstrated decreased alkaloid concentration in the stems with increasing maturity. Alkaloid concentration tended to increase with maturation in both leaves and roots, then leveled off at the final harvest when all of the plants were in full bloom. In other words, the maximum concentration appeared during the flowering stage. The decreases in alkaloid concentration at the fourth harvest of 45% in the leaves and 39% in the roots were considered significant. Total alkaloid content of the leaves and roots generally followed the pattern exhibited with alkaloid concentration, however, the total alkaloid content of the stems was more directly related to the effect of GA on increasing stem growth. Due to significantly increased stem weight the total alkaloid content of this organ was markedly increased at all three harvests. The increases of 75% at the second harvest and of 52% at the third harvest were considered significant.

Of great interest was the disclosure of the effect of GA on the concentration of VLB and the total VLB content. It was concluded that the stems and roots of both groups either contained none of this alkaloid, or that this alkaloid, if present, was present below the minimum detectable concentration that could be demonstrated by the assay procedure. Treatment with GA generally increased the concentration and total content of VLB in the leaves. The concentration of VLB in the leaves of this group decreased with increased plant maturity. This pattern was the reverse of that noted with the concentration of total leaf alkaloids. Even in the control group the concentration of VLB in the leaves was about two-thirds that of the first harvest at the terminal harvest. These disclosures are in agreement with the observations by Noble (12) who found that extracts of leaves of young *C. roseus* plants showed a greater leukopenic activity than did the leaves of older plants. This was undoubtedly due to a lower concentration of the leukopenic alkaloid VLB in the older leaves. This suggests that VLB accumulates at a higher rate in younger leaves than in more mature ones. The total VLB content of the leaves followed a pattern similar to that of VLB concentration since gibberellin treatment did not markedly affect leaf growth.

The following pertinent observations were made concerning other types of metabolic products. Treatment with GA apparently did not affect the chlorophyll concentration. The synthesis and/or accumulation of lipids and resinous compounds was reduced in all of the organs of the treated groups as indicated by the decreased quantities of petroleum ether and ether extracts obtained by the selective solvent extraction. No significant differences were noted in the alcoholic or aqueous extracts.

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Keyphrases

Catharanthus roseus plants
Gibberellic acid effect—growth, alkaloid content
Vincaleukoblastine content—gibberellic acid
Chlorophyll content—leaves