The Gibberellic Acid Inhibition of Nicotine Biosynthesis

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Parups (1959, 1960) has shown that the reduced nicotine content of tobacco plants treated with gibberellic acid (cf. Yabuta *et. al.*, 1943) is due to a reduced rate of synthesis and not to an accelerated destruction of nicotine in the plant body. This conclusion was validated in *Nicotiana rustica* for both aerial organs and excised roots in sterile culture.

The observations of Parups have been of considerable interest in connection with our studies of the intermediates in nicotine synthesis. We have found that nicotinic acid can provide the pyridine ring (1960), and Dewey, Byerrum and Ball (1955) and Leete (1955) have found that ornithine provides the pyrrolidine ring. It is an interesting fact, however, that additions of nicotinic acid and of ornithine, separately or together, do not influence the rate at which nicotine is made by excised tobacco root cultures as long as growth rate is not affected. We have concluded that the biosynthesis possesses not only substantial rate stability but also a rate dependency on growth. Obviously, it would be experimentally advantageous if means were available for altering these rate relationships. The observations of Yabuta *et al.* (1943), of Parups (1960), and of Burk and Tso (1958) appear to indicate the possibility for such alteration.

In this paper we confirm the reported effects of gibberellic acid on nicotine production and degradation by excised tobacco root cultures. It is shown by the use of labeled precursors that gibberellic acid affects the incorporation of exogenous precursors to a limited extent and that the overall effect is principally upon utilization of endogenous precursors in the biosynthesis. Further, it is shown that the inhibitory action of gibberellic acid very likely occurs at three different points in the metabolic sequence. Two of these are involved in production of ornithine and of nicotinic acid, respectively. The third lies between nicotinic acid and ornithine on the one hand and nicotine on the other. It is in the latter area that the rate-limiting steps for both growth and nicotine formation are thought to occur (Dawson, 1960).

Materials and Methods

Root cultures: Excised root cultures of *Nicotiana tabacum L.* var. Turkish were grown by methods described elsewhere (Solt, 1957). One clone derived initially from a single seedling was used in all experiments.

Gibberellic acid: Two preparations of gibberellic acid were used. One was kindly supplied by Dr. Louis Nickell of the Charles Pfizer Company. The other was a commercial preparation (75%) purchased from Distillation Products Industries. The action of both preparations was the same.

Labeled intermediates: Nicotinic acid-2-H^3 (216,000 dpm/mg) was prepared from 2-bromo-3-picoline by methods described elsewhere (Dawson *et al.*, 1960). Nicotine-2-H^3 was prepared by feeding nicotinic acid-2-H^3 to excised root cultures and isolating the nicotine produced. Specific activity of the nicotine was 44,500 dpm/mg. For details of location of label see Dawson, Christman, Solt, D'Adamo and Wolf (1960). DL-Ornithine-2-C^{14} was purchased from Volk Radiochemical Company.

Addition of precursors: In general, cultures in lots of 25 or 50 were incubated for one week prior to the addition of both the precursor and gibberellic acid. Nicotine-2-H^3, ornithine, and gibberellic acid were filter sterilized through Pyrex fritted glass disks (UF). Nicotinic acid was sterilized by autoclaving. Material was pipetted aseptically to the root cultures, and incubation was continued for an additional four weeks.

Isolation and assay of nicotine: The roots were harvested as described earlier (Dawson *et al.*, 1960). For assay both the dried root tissue and the concentrated spent culture solution were made basic with MgO and steam distilled. The alkaloid was removed from the distillate by an Amberlite IRC-50 resin column. The nicotine was eluted from the columns with 25 ml of N HCl and the amount estimated spectrophotometrically by the method of Willits, Swain, Connelly and Brice (1950) using physical constants established in this laboratory.

For isolation of nicotine as a picrate the alkaloid was steam distilled from an excess of MgO into an excess of aqueous picric acid. The volume of the distillate was reduced under reduced pressure, the crystals redissolved by boiling and nicotine dipicrate separated by crystallization.

Radioisotope assays: C^{14} and H^3 were assayed by combustion of nicotine dipicrate and proportional gas counting (Christman *et al.*, 1955; Christman and Wolf, 1955; Wilzbach *et al.*, 1953; Christman, 1957). Results are expressed as radiochemical yield, that is, the proportion of radioactivity originally supplied to

the cultures which was recovered in the form of nicotine. Ratios of specific activity are also given. These ratios indicate the proportion of nicotine which was made from labeled precursors.

Nicotinic acid assay: Total acid-hydrolyzable nicotinic acid of the roots and spent culture fluids was assayed by a modification of the method of the Association of Vitamin Chemists (1947). The bioassay procedure uses *Lactobacillus plantarum* (Orla-Jensen) Holland, strain 17-5 as test orgauism.

Results

Reduction of nicotine yield: When gibberellic acid was added to the culture medium 29 days before harvest at a final concentration of 0.5 to 20 ppm, a small stimulation of root dry weight production and a marked inhibition of nicotine output were observed. The maximum effects **(Table 1)** were obtained at a coucentration of 1 ppm gibberellic acid. Higher concentrations gave no additional effects. Similar results were obtained by adding the equivalent of 5 ppm of gibberellic acid at each of several five day intervals **(Table 2)** during the culture passage.

Nicotine destruction: The presence of gibberellic acid was not associated with an accelerated rate of destruction of added nicotine **(Table 3)**. The fact that substantial loss of radioactivity occurred from nicotine-2-H^3 is evidently a reflection of the lability of the tritium atom in ring position 2 under the conditions of root culture and of alkaloid isolation. Earlier experiments (Dawson *et al.*, 1960) have shown that nicotine labeled with C^{14} in the pyridine ring may be recovered from these Turkish tobacco root cultures with approximately 90% radiochemical yield. The essential point in the present connection is that gibberellic acid did not induce a further lowering of the radiochemical yield when tritium-labeled alkaloid was supplied to the cultures.

Incorporation of labeled intermediates: When nicotinic acid-2-H^3 was supplied to the root culture for 29 days, simultaneous addition of gibberellic acid substantially reduced the chemical yields of nicotine **(Table 4)**. Owing to the higher specific activities of nicotine produced in the presence of gibberellic acid, however, the radiochemical yields were similar.

In earlier work (Dawson *et al.*, 1960a), we have observed substantial (up to 50%) losses of added nicotinic acid in the course of the usual

Table 1. Effects of different amounts of gibberellic acid, added at one time, upon dry weight and nicotine production by excised roots of Turkish tobacco in sterile culture. Duration of experiment, 29 days.

Gibberellic acid concentration	Root dry weight	Nicotine yield	Relative nicotine yield
ppm	mg	ug	ug/mg
0	25.9	1113	42.9
0	24.8	994	40.1
0.5	27.9	756	27.0
1	30.0	693	23.1
5	31.8	731	22.9
10	29.1	678	23.2
20	31.2	693	22.2

Table 2. Effects of different amounts of gibberellic acids, added serially at five-day intervals, upon dry weight and nicotine production by excised tobacco root cultures. Duration of experiment, 29 days.

Gibberellic acid serial additions	Root dry weight	Nicotine yield	Relative nicotine yield
ppm	mg	ug	ug/mg
0	27.2	1246	45.8
0	23.7	1178	49.7
5	24.9	606	24.3
5+5	27.1	672	24.7
5+5+5	25.0	638	25.5
5+5+5+5	22.7	508	22.3

Table 3. Effect of gibberellic acid (5 ppm) upon the recovery of radioactivity from added nicotine-2-H^3. Each lot received 1.64 mg with an activity of 44,544 dpm/mg. Duration of experiment, 27 days.

Treatment	Nicotine yield*	Radiochemical yield
	mg	%
Nicotine-2-H^3	238	67
"	232	62
Nicotine-2-H^3 plus Gibberellic acid	138	64
"	143	68

* *As dipicrate. Corrected for solubility.*

Table 4. Effects of gibberellic acid (5 ppm) upon the incorporation of nicotinic acid-2-H^3 (0.6 mg. per culture) into nicotine. Duration of experiment, 29 days.

Treatment	Nicotine yield[a]	Specific activity[a]	Radiochemical yield	Ratio of activities[b]
	mg	dpm/mg	%	%
Nicotinic acid	86	3694	9.4	8.6
" "	82	3680	9.3	8.6
Nicotinic acid plus Gibberellic acid	38	6743	7.6	16.1
	45	7725	9.3	18.0

[a] *As the dipicrate.*
[b] *Calculated on a common weight basis and on the assumption that all label is in the pyridine ring of nicotine.*

Table 5. Effects of gibberellic acid (5 ppm) upon utilization of nicotinic acid (500 ug per culture) by excised tobacco roots. Duration of experiment, 27 days.

Treatment	Root dry weight	Nicotine yield	Nicotinic acid in medium	Nicotinic acid in roots
	mg	ug	ug	ug
No gibberellic acid	22.2	1262	268	2.1
" "	21.4	1154	282	2.1
Gibberellic acid	22.9	587	209	2.8
" "	22.6	604	220	2.9

Table 6. Effects of gibberellic acid (10 ppm) upon the incorporation of ornithine-2-C^{14} (0.09 mg. per culture) into nicotine by excised tobacco root cultures. Duration of experiment, 28 days.

Treatment	Nicotine yield[a]	Specific activity[a]	Ratio of sp. activities[b]	Radiochemical yield
	mg	dpm/mg	%	%
No gibberellic acid	115.4	2.90×10^4	13.7	15.8
" " "	110.5	3.00×10^4	14.2	16.9
Gibberellic acid	58.5	4.25×10^4	20.1	11.9
" "	51.7	4.05×10^4	19.1	11.5

[a] *As the dipicrate.*
[b] *Calculated on a common weight basis on the assumption that all label is in the pyrrolidine ring.*

Table 7. Effects of gibberellic acid (10 ppm), with and without added ornithine (0.5 mg per culture), upon the dry weight and nicotine yields of excised tobacco roots in sterile culture. Duration of experiment, 21 days.

Treatment	Root dry weight	Nicotine yield	Relative nicotine yield
	mg	ug	ug/mg
No additions	18.4	907	49.2
" "	19.1	915	47.9
Gibberellic acid	21.5	482	22.4
" "	24.6	595	24.1
Gibberellic acid plus ornithine	25.3	566	22.3
	29.8	518	23.7

experimental period. On the possibility that gibberellic acid may have accelerated markedly the rate of disappearance of nicotinic acid from the system and thus led to the development of a rate-limiting concentration of this precursor, microbiological assays were performed upon root tissues and spent culture fluids. The results obtained when cultures had been supplied with ca. 0.5 mg each of nonlabeled nicotinic acid are shown in **Table 5**. Gibberellic acid induced the usual 50% drop in chemical yield. However, the total amount of nicotinic acid remaining in the medium was lowered only slightly by gibberellic acid, while the nicotinic acid content of the root tissues was somewhat increased. It seems clear, therefore, that the reduced nicotine yields are not a result of reduced concentrations of nicotinic acid precursor.

When labeled ornithine was supplied to the cultures with and without gibberellic acid, the results were qualitatively the same **(Table 6)**. Nicotine yield was again reduced up to 50% by gibberellic acid. The specific activity of nicotine was also increased although not so much as in the case of nicotinic acid feeding. Furthermore, in the presence of gibberellic acid, radiochemical yields were somewhat reduced.

Again, to test the possibility that gibberellic acid may have led to rate-limiting concentrations of ornithine by accelerating its catabolism, substantially higher concentrations (0.5 mg per culture) of the nonlabeled amino acid were added to the culture medium in a separate experiment **(Table 7)**. In this case also, the addition of ornithine had no effect upon the extent of the reduction of nicotine yields by gibberellic acid.

Discussion

Clearly, in this experimental system, gibberellic acid may reduce nicotine yield up to 50% without exerting a pronounced influence upon growth rate. In fact, the general trend is towards slight growth stimulation. It is interesting to note that the effects are achieved at very low concentrations of gibberellic acid and that above these concentrations the response is flat. It would appear that effective concentrations of the acid are readily maintained in such cultures. That is, comparable results were obtained when the acid was added to culture medium in fractional amounts throughout the experimental period and when it was added in a single batch at the beginning of the experimental period.

Perhaps the most interesting result of this work is the observation that a reduction of approximately 50% in nicotine yield from gibberellic acid feeding was not reflected in a decreased radiochemical yield when ring-labeled nicotinic acid was supplied as a nicotine precursor. The reductions in chemical yield were exactly counterbalanced by the increases in radiochemical yield.

Labeled ornithine was supplied with gibberellic acid with similar results. There was the usual 50% reduction of nicotine yield, but the increase in specific activity of nicotine resulting from gibberellic acid feeding was not as great **(Table 6)** as was the case when labeled nicotinic acid was supplied *(see above)*. The radiochemical yield was actually decreased somewhat in the presence of gibberellic acid.

The foregoing results may be given a unified explanation by assuming that dilution of endogenous precursor pools at the sites of syn-

thesis was achieved to a different extent in the case of each added intermediate. The mean extent of such dilution, as estimated by the ratios of specific activities of products to precursors (**Tables 4 and 6**), in the case of labeled nicotinic acid was about 9% in the absence of gibberellic acid and 18% in its presence. In the case of labeled ornithine, the corresponding figures are 14% and 20%, respectively. If the endogenous precursors are, in fact, nicotinic acid and ornithine, and if there is no compartmentalization of endogenous and exogenous precursor pools or pathways, then these results indicate that the mode of action of gibberellic acid must involve an inhibition of one or more steps in the metabolic sequences involved in the formation of both nicotinic acid and ornithine.

In part, these results appear to agree with the interpretations of Parups. However, the fact that addition of an excess of either precursor fails to affect the inhibitory action of gibberellic acid indicates that the nature of the inhibition is not as simple as Parups suggests. Rather, it would appear that gibberellic acid has still another effect, namely, the inhibition of a third step in the metabolic sequence located somewhere between the two precursors used in these experiments and the completed nicotine molecule. It is interesting to note that the latter sector of the overall metabolic sequence is also the general locus of the rate-limiting step for nicotine biosynthesis under normal circumstances (Dawson, 1960). It is also considered to be the locus of the rate-limiting step for growth of Nicotiana root cultures (Dawson, 1960).

If it be assumed that the inhibitory action of gibberellic acid is chemical rather than physical in nature, then two alternative conclusions may be drawn. First, gibberellic acid acts at several different steps in the metabolic sequence rather than only one and is hence not highly specific in its action. Second, the acid may be a relatively specific inhibitor for a fundamental reaction which is widely employed in cellular biochemistry. Further experiments are needed to permit a choice between the alternatives.

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