CORRELATION BETWEEN INACTIVATION OF 2,4-DICHLOROPHENOXYACETIC ACID & CESSATION OF CALLUS GROWTH IN BEAN STEM SECTIONS[1]

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In a previous publication (1) we have described an in vitro system for studying the callus growth on bean stem sections which is initiated by applying relatively high concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D). Only short exposure to 2,4-D was necessary to initiate the callus growth. Since this growth is not tumorous in nature and therefore not auxin autonomous, it will continue only as long as there is a supply of the growth inducing material still present in the tissue. It was of interest, therefore, to follow the fate of C^{14}-labeled 2,4-D in this system and to attempt to correlate it with the data on callus growth. This paper will report the results of these experiments.

METHODS & MATERIALS

The methods for harvesting bean stems and growing them aseptically on nutrient agar were the same as previously described, except that the amount of agar in the petri plates was kept constant, so as to control the depth to which the stems were implanted. In all cases exposure to 2,4-D was for 24 hours except in the CO_2 trapping experiments, where the 2,4-D blocks were kept in place for the full duration of the experiment. Because the amount of radioactivity which was secreted into the medium from the basal ends of the stems was very low, it was necessary to concentrate large amounts of medium to obtain accurate estimates of the amount of radioactivity present and to determine the nature of the radioactive compounds involved. Since the agar medium did not lend itself to concentration and subsequent paper chromatography, a method for obtaining proliferations in liquid medium was developed. For this purpose, specially constructed stainless steel holders were used to maintain the stem sections in a vertical position with their lower ends in the liquid medium. The agar blocks were placed on top of the stems while the stems and holders were placed in an empty, sterile petri plate to avoid contaminating the medium with radioactive 2,4-D should the agar blocks drop off in the course of the manipulations.

The assembled units (holders, stems, & 2,4-D blocks) were then transferred to new sterile petri plates or other containers and the medium added. When the evolution of $C^{14}O_2$ from C^{14}-labeled 2,4-D was studied, the holders were placed in 100 ml beakers which had been cut off to a height of 25 mm. Sixteen stems were placed in each beaker along with 10 ml of medium. The beakers were closed with rubber stoppers which had been cut to a thickness of 10 mm and sealed with a beeswax-rosin mixture. The rubber stoppers had two glass tubes leading through them. One tube was connected to a source of sterile, humidified, CO_2-free air, and the other tube to a bubbler in a 12 ml centrifuge tube for trapping the evolved CO_2 in 15% KOH solution. Eight such beakers were arranged in parallel and shared the same source of treated air. The flow of air through each beaker was regulated to keep the bubbling rate in the CO_2 traps constant. The recovery of respired CO_2 was checked with yeast cultures respiring C^{14}-labeled glucose in this system. Recoveries of 95% were obtained routinely.

At the end of the experiment, the trapped CO_2 was precipitated by adding a few drops of 12% $BaCl_2$. A check for completeness of precipitation was made after the precipitated $BaCO_3$ was removed by centrifugation. The agar blocks for each vessel were collected and pooled. They were melted by heating with a few drops of water, adjusted to volume, and aliquots counted. The medium was concentrated under vacuum and the stems were extracted with alcohol. Aliquots of the extract, the fibrous residue, and the concentrated medium were counted. Separate aliquots of the stem extracts and the concentrated medium were also spotted on chromatograms and developed as described (1).

RESULTS & DISCUSSION

Figure 1 shows the percent increase in fresh weight of bean stem sections which received one and two consecutive applications of 2,4-D. Each point represents the average of five replicates, each of which

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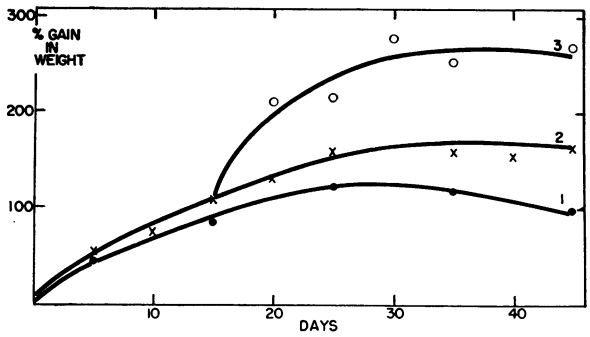

FIG. 1. Effect of reapplication of 2,4-D on fresh weight of bean stem sections. Curve 1, control. Curve 2, 10^{-4} M 2,4-D agar blocks applied for 24 hours at beginning of experiment. Curve 3, 10^{-4} M 2,4-D agar blocks applied for 24 hours at beginning of experiment and again after 15 days.

consisted of five stem sections. Controls were treated identically to exposed stems except agar blocks free of 2,4-D were applied. It will be noted that a second application of 2,4-D, after growth from the first application had slowed down, resulted in a new spurt of growth, equal in magnitude to the first. It would appear, therefore, that the cessation of growth in this system was not due to the exhaustion of any nutrients in the medium but rather to the exhaustion of the growth inducer, 2,4-D.

As mentioned previously (1) it was not possible to obtain proliferation in bean stems floated on a liquid medium. Further investigation indicated that the proliferation process was strongly aerobic and that the floated sections apparently did not receive enough oxygen. Similarly, when stems were supported in the specially designed holders in closed vessels, no growth was observed in the absence of circulating air, even when CO_2 was absorbed by KOH in center wells. If, however, the beakers were continuously aerated, growth comparable to that observed on agar was obtained.

Table I summarizes the fate of the radioactivity from C^{14}-carboxyl labeled 2,4-D in the course of a growth experiment on liquid medium. Each value is the average of four individual determinations, using 16 bean stems in each determination. Note that the amount of radioactivity liberated as $C^{14}O_2$ was quite low, and seemed to increase only toward the end of the experiment. In spite of all efforts to maintain aseptic conditions, it did not prove feasible to completely prevent contamination of the medium. It is very possible, therefore, that the apparent increase in $C^{14}O_2$ liberation toward the end of the experiment is actually due to bacterial oxidation of the radioactive metabolites of 2,4-D which were slowly released into the medium. The apparent failure of the radioactivity in the medium to increase in the latter part of the experiment also agrees with this explanation. Most of the radioactivity which had been taken up by the stems was in an ethanol-soluble form in the stems at the end of the experiment. Paper chromatographic analysis of these extracts revealed that the radioactivity was almost exclusively in the band at R_f 0.5 in Butanol-propionic acid-H_2O (8:5.6:12 v/v/v) (3). The radioactivity in the medium, on the other hand, moved near the front in this solvent, as did 2,4-D. There was only little if any binding of radioactivity to the ethanol-insoluble residue of

TABLE I

BALANCE SHEET OF FATE OF C^{14}-CARBOXYL LABELED 2,4-D IN BEAN STEM SECTIONS

FRACTION	% OF TOTAL APPLIED RADIOACTIVITY		
	1ST DAY	3RD DAY	7TH DAY
Agar blocks	53±5	24±0.4	23±0.4
Bean extracts:			
R_f 0.5 Complex	16.6±1.5	42.8±1.8	46.5±1.8
Free 2,4-D	27.4±2.5	19.2±0.8	11.5±0.4
Medium	1.63±0.06	5.5±0.6	5.7±0.2
Respired CO_2	0.76±.02	3.5±0.02	8.3±0.3
Fiber residue	1.51±0.09	4.4±0.4	4.6±0.3
Total recovery	106±6.3	101±4.3	97.2±4.0

TABLE II

CORRELATION BETWEEN GROWTH & INACTIVATION OF APPLIED C^{14} LABELED 2,4-D

TIME DAYS	% GAIN IN FRESH WT		ETHANOL EXTRACT cpm/TOTAL SAMPLE	DISTRIBUTION OF RADIOACTIVITY (%)	
	2,4-D TREATED	CONTROL		R_f 0.5 COMPLEX	2,4-D
2	44±3	3.2±0.9	67.4±2.1	44	55
4	71±4	27±3	74.6±6.0	72	28
7	142±35	29±18	64.4±3.3	87	13
8	95±11	23±3	69.9±3.7	87	13
10	144±16	67±6	58.0±3.5	89	10
15	176±17	58±14	60.7±5.6	88	12
21	188±21	106±4	62.4±5.2	97	3

the cells. These results are largely in agreement with those obtained by Weintraub et al (4) with whole bean plants.

The radioactivity from 2,4-D remained in the stems for very considerable lengths of time (table II). In this experiment the growth [weight gain expressed as (final wt-initial wt) × 100/initial wt] of the stems was measured along with the radioactivity which remained in them in ethanol-extractable form. Paper chromatography of the extracts revealed that the compounds moving at R_f 0.5 were essentially the only detectable source of radioactivity after the 15th day of incubation. Thus there appears to be a good correlation between the disappearance of detectable amounts of free 2,4-D and the cessation of growth. Apparently the compounds moving at R_f 0.5 can be regarded as true detoxification products of 2,4-D in that they remain in the tissue but appear to exert no effect on it. Hay and Thimann also have implied the presence of such a detoxification product in bean seedlings (2). It will be of interest to verify this point by direct experimentation. For this purpose purified preparations of the compounds of R_f 0.5 are required since crude isolates of this material appeared to be quite toxic to the tissues and inhibited all growth in bioassays. The purification of this material and studies of its chemistry and biological effects will constitute the subject of further publications.

Summary

Results have been presented which correlate the cessation of growth of excised bean stem sections treated with 2,4-D, with the disappearance of free 2,4-D from the sections. The radioactivity from C^{14}-carboxyl labeled 2,4-D largely remained in the tissue in an ethanol-soluble form chromatographically distinct from 2,4-D and only relatively small amounts of radioactivity were found in CO_2 or released into the medium.

Acknowledgments

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