Pyrimidine Pathway in Boron-deficient Cotton Fiber

IRENE M. WAINWRIGHT, RAYMOND L. PALMER, AND W. MACK DUGGER[1]
Department of Botany and Plant Sciences, University of California, Riverside, California 92521

ABSTRACT

Cotton ovules cultured in an insufficiency of boron (10 micromolar), showed inhibition of fiber growth by the ninth day in culture. Averaging data from eight to eleven days of culture under these conditions, total incorporation of [6-^{14}C]orotic acid into fiber was inhibited by 59%. Inhibition was evident in all radioactively labeled pools, indicating that the effect may be at the membrane transport level or at an early stage of orotic acid metabolism. On a per cent basis, incorporation into RNA under boron deficiency was higher than under sufficiency. The effect is greater on the eighth day of culture, with a decreasing difference from controls up to the eleventh day. Conversely, the per cent incorporation into UDP-glucose was lower under boron deficiency than in controls, having a more or less constant value from 8 to 11 days of culture. Thus, a primary event of boron deficiency in cotton fiber culture is an alteration in the flow of metabolites through the pyrimidine synthesis pathway.

A possible primary event of boron deficiency is the influence of boron on pyrimidine metabolism. A number of investigators report that RNA levels are depressed under boron deficiency, particularly at the apical meristems (26, 29). However, Cory et al. (8) found that ^{32}P-inorganic phosphate incorporation into nucleic acids of *Vicia faba* root sections was greater under boron deficiency than in controls if the experiment was done before visible injury was apparent. This result was confirmed by Robertson and Loughman (18, 19) for *Vicia* roots where elongation was being inhibited but had not ceased. In recent studies, Chapman and Jackson (6, 13), working with *Phaseolus* root tips, showed both early and late effects. There was an early (6–10 h) increased uptake of [^{14}C]-uridine or [^{32}P]inorganic phosphate into RNA at a time when the RNA levels were unchanged. RNA levels fell only after root elongation ceased (100 h) concurrently with manifestation of other boron deficiency symptoms. Increased ribonuclease activity may accompany the decrease in RNA in boron-deficient tissues (6, 25).

Information about the influence of boron on nucleotide levels is very sparse (4, 5, 28). Robertson and Loughman (18, 19) found that the per cent [^{32}P]inorganic phosphate incorporated into nucleotides was lower under boron deficiency than sufficiency. Teare (26) found that there was a general decrease in [^{32}P]inorganic phosphate incorporation into nucleotides in boron-deficient *Phaseolus* roots compared to controls. However, there was a 200-fold increase in UTP and a corresponding 300-fold decrease in UDP-glucose. These data seem to correlate with the finding (9) that *in vitro* UDP-glucose pyrophosphorylase is stimulated by the addition of boron. It follows that formation of UDP-glucose and therefore cell wall synthesis and other synthetic reactions requiring UDP-glucose might be inhibited by boron deficiency. Birnbaum et al. (3) hypothesized that the resultant accumulation of UTP

might account for the enhanced RNA incorporation of pyrimidine nucleotide precursors as discussed above.

Working with the *in vitro*-cultured cotton fiber system, Birnbaum et al. (21) found that the OMP^2-decarboxylase inhibitor, 6-azauracil gave symptoms resembling those caused by boron deficiency (3). The correlation was further strengthened by the finding that low uracil concentrations partially overcame both boron deficiency and 6-azauracil effects, presumably by utilization of the uracil salvage pathway.

In the present study, we sought to establish more directly whether boron regulates the pyrimidine pathway in some way. Our experimental tissue was fiber from the cotton ovule culture system. We studied the incorporation of [^{14}C]OA into intermediates of the pyrimidine pathway. A factor which had to be taken into account was the possible effect of boron deficiency on OA uptake by the tissue itself.

A definitive study on the interaction of boron with ion transport mechanisms has been done by Loughman's group (14, 16–19), using mainly *V. faba* seedlings at a stage where root elongation was inhibited by boron deficiency but had not totally ceased. They discovered that the reduced uptake of [^{32}P]phosphate and other inorganic ions caused by boron deficiency, actually involves several components including translocation, metabolism, interaction with auxin, and transport across the cell membrane. The depressed membrane transport under boron deficiency is considered to be a reflection of the role of boron in maintaining the conformation of certain membrane components. Pollard et al. (16) have shown that the proposed membrane phosphate carrier, ATPase, has lower activity under boron deficiency conditions but can be activated by addition of boron.

MATERIALS AND METHODS

Cotton plants (*Gossypium hirsutum* L., Acala SJ-1) were grown in the greenhouse, and the ovules were cultured *in vitro* on the day of flower anthesis according to the method of Beasley and Ting (1) and Birnbaum et al. (2). Special 125-ml Bellco, boron-free glass flasks were used for the culture, and the medium contained 100 μM boron (control) or 10 μM boron (experimental).

After 9 days, cultures were prepared for introduction of the [^{14}C]OA; this involved removing medium from the culture flask to leave a known volume of 20–30 ml/flask. To this we added [6-^{14}C]OA (50 mCi/mmol, Calatomic; or 25 mCi/mmol, ICN). Sterile technique was used, and care was taken not to disturb the ovules more than necessary. Cultures were initially shaken carefully to prevent the ovules from sinking below the surface of the culture solution and then incubated at 34 C for up to 4 h.

After the allotted incubation time, 10 ovules were selected from each flask, and then washed, blotted dry, and frozen on dry ice. The fibers were removed while still frozen and ground to a powder with a mortar and pestle and a small quantity of sand. The subsequent procedure (15) involved extracting with 3 ml 5% trichloroacetic acid (containing 0.05% 8-hydroxyquinoline), centri-

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[1] To whom reprint requests should be addressed.

[2] Abbreviations: OMP: orotidine monophosphate; OA: orotic acid.

fuging at 20,000g for 5 min, washing the pellet with 3 ml extraction solution, and recentrifuging. The combined precipitates were incubated with 0.3 M potassium hydroxide to solubilize RNA following the procedure of Ross (20, 22). Total incorporation was calculated by the sum of the values for all the fractions. The acid-soluble fraction (the trichloroacetic acid supernatant) was extracted with ether (15) and freeze-dried.

Paper chromatography of the ether-treated acid-soluble fraction was carried out as described by Cole and Ross (7). Standard nucleotides and their derivatives were located by fluorescence under UV light, and catabolic pathway components were located by the staining methods described by Ross (22). Autoradiography was used to locate chromatographic spots from fiber extracts. Six weeks to 3 months were allowed for latent images to develop on x-ray film (Kodak blue sensitive 4527).

The identified areas from the chromatograms were cut out and diluted in 0.1 N hydrochloric acid for counting. The spots were positively identified by co-chromatography with standards. The catabolic pathway components that co-chromatographed (except uridine) were separated by rechromatography, using BAW (n-butanol-acetic acid-water, 2:1:1) in the first dimension and Pabst solvent III in the second (22).

RESULTS

Ovule Development in Culture. Of several boron concentrations, 10 μM was found to produce fiber growth characteristics most suited to the purposes of studying boron deficiency effects. At this concentration, fiber lengths were the same as the control (100 μM) up to the 9th day of culture, but growth slowed thereafter and was completely inhibited at some point between 11 and 13 days (Fig. 1). Concentrations of boron decrementally lower than 10 μM resulted in successively earlier inhibition of fiber growth (data not presented). Using the 10-μM boron level, we hoped to restrict this study to fiber metabolism only and not to include fiber versus the ovule callus growth observed with completely boron-deficient ovules (2). It is apparent that there is a shift from boron sufficiency
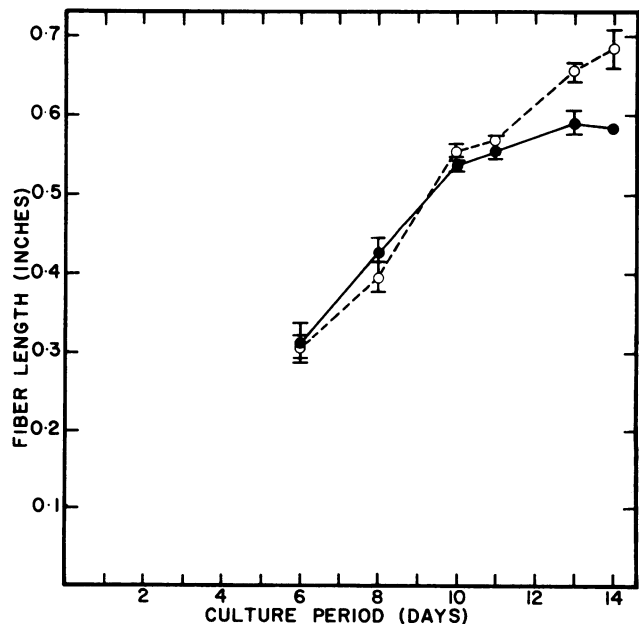
to deficiency at, or just before, the 9th day. Thus, by using ovules of this culture age, we hoped to obtain data about a primary event in boron deficiency. In several experiments where boron was resupplied at the 9th day, growth inhibition could be reversed.

The fiber weight data confirm the boron insufficiency effect of supplying 10 μM boron rather than 100 μM boron (data not shown). Ovule body dry weight followed the same pattern except that the boron-insufficient cultures had higher values than the controls after the 10th day. This was a result of greater callus growth under boron deficiency (2).

Radioactive Incorporation into Major Pools. By the 8th to 11th day of culture, total incorporation of [6-^{14}C]OA into the fiber was partially inhibited in a medium containing 10 μM boron (Table I). Averaging 18 data sets, 59% inhibition of OA incorporation occurred under boron deficiency. The majority of the counts appeared in the acid-soluble fraction (90.3% ± 2.4 and 89.7% ± 2.0 for the 100 μM and 10 μM boron cultures, respectively). The RNA fraction also had depressed incorporation compared to the controls (Table I), but inhibition was only 44%. The RNA fraction contained less than 10% of the total counts (7.9% ± 1.3 and 8.4% ± 1.4 for 100 μM and 10 μM boron, respectively). Counts in the residue fraction were negligible, usually less than 2%. Total incorporation and incorporation into the acid-soluble and RNA fractions were linear over 4 h after a lag during the first 0.5 h.

In one experiment, incorporation of [6-^{14}C]OA was measured on the 8th, 9th, and 11th days of the culture period. With 100 μM boron, there were only slight daily variations in incorporation into both the RNA and acid-soluble fractions. On the other hand, for fibers grown in 10 μM boron, there was a marked increase in inhibition of incorporation into both fractions on the 9th and 11th day compared to the 8th (Table II). Thus, there was a metabolic as well as morphological change during this phase of the culture period, indicating a transition from boron sufficiency to deficiency.

Chromatography and Incorporation into Pyrimidine Pathway Intermediates. Prior to chromatography, the acid-soluble fraction was extracted with ether, and some counts were lost in the ether fraction, the amount depending on the particular experiment. To examine this further, we added various radioactivity labeled intermediates during homogenization. There was little variation in fractionation between the ether and water phases of the different compounds (OA, uracil, UTP, and UDP-glucose). In this experi-

FIG. 1. Fiber growth of cotton ovules cultured with (O- - -O) 100 μM boron, or (●——●) 10 μM boron. Each point is the mean of the fiber lengths of 30 ovules. Bar represents 1 SE above and below the mean. Only on day 13 were the means significantly different at the 5% level (t test). The measurement taken for each ovule was the mean length of the longest fibers.

Table I. *Ratio of Incorporation of [^{14}C]OA into Fiber from Cotton Ovules, Cultured in 10 and 100 μM Boron*

Ovules were grown for 8–11 days, and one to five flasks were used per treatment (10 ovules/flask). Incubation was in 0.025–0.400 μCi/ml [6-^{14}C]OA.

	Incorporation for 10:100 μM Cultures
Total incorporation	0.41 ± 0.07[a]
Incorporation into acid-soluble fraction	0.42 ± 0.03
Incorporation into RNA	0.56 ± 0.08

[a] Mean of 18 experiments ± 2 SE.

Table II. *Ratio of Incorporation of [^{14}C]OA into Fiber of Cotton Ovules Cultured in 10 and 100 μM Boron, Measured on 3 Days of the Culture Period*

Mean of three flasks for each measurement. Incubation was for 4 h with 0.5 μCi [6-^{14}C]OA/flask, in 20 ml culture medium.

	Time of Culture		
	8	9	10 days
RNA	0.49	0.26	0.36
Acid-soluble fraction	0.38	0.13	0.27

ment, the RNA fraction contained negligible radioactivity but the residue had what was obviously nonspecifically bound radioactivity. This value was fairly constant and independent of the amount of applied radioactivity and the type of compound.

Figure 2 shows the paper chromatographic separation of a radioactive fiber extract. UMP and OA were not separated in the chromatography of plant extracts. The fact that radioactive OMP was never found on the chromatograms from plant extracts, confirms the view that the OMP pool size is very small (22). The unknown spots (UK-1, 2, and 3) appear to correspond with the unknowns obtained by Ross and Cole (23), while the catabolic pathway components (uracil, β-alanine, β-ureidopropionic acid, and dihydrouracil) co-chromatographed in this solvent system. On elution and rechromatography we found that β-alanine was the major component (68–82%). Incorporation into the various metabolites was found to be linear over 4 h.

We attempted to distinguish any effects on particular components of the pyrimidine pathway from the general inhibition of uptake during boron deficiency by expressing the data on a percentage basis (Table III). The UMP + OA data were omitted in these calculations because there were indications that a nonincorporated OA (resulting from incomplete washing) was extracted into the trichloroacetic acid fraction. Also omitted were the compounds present in small amounts, such as the cytidine compounds. Uridine and the rest of the catabolic components were combined under one category termed the "catabolic pathway" compounds.

Table III gives the data for a representative experiment. The variation between experiments was in part due to the variability in the sensitivity of ovules to boron deficiency at different seasons
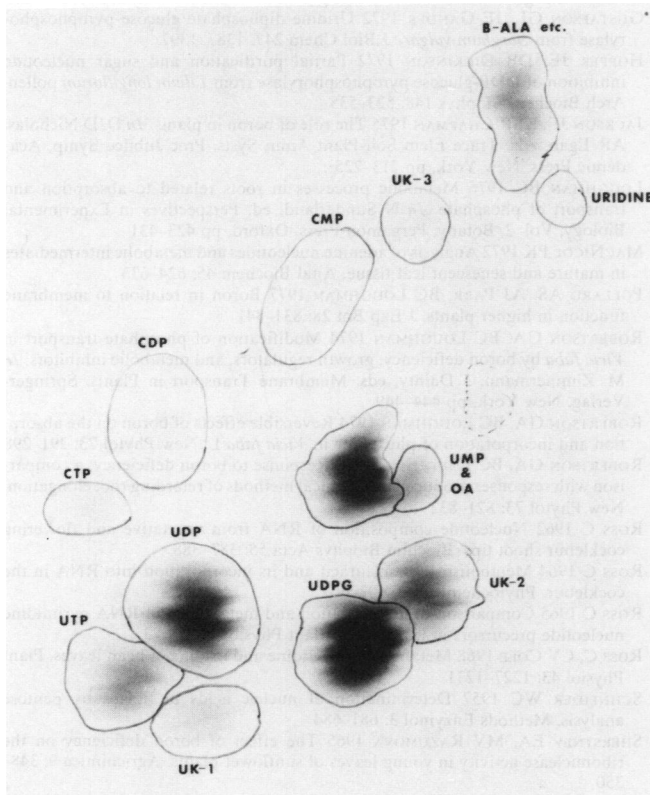
Table III. *Mean Per Cent Incorporation of [¹⁴C]OA into Major Intermediates in the Pyrimidine Synthesis Pathway*

The data are the mean ± 2 SE.

Compounds	Boron Concentration	
	100 μM	10 μM
RNA	6.9 ± 0.6[a]	12.4 ± 1.3
UDP-glucose	63.3 ± 1.6	49.1 ± 2.6
Catabolic pathway	3.5 ± 0.2	5.1 ± 0.5
UDP	23.5 ± 0.6	28.6 ± 1.3
UTP	2.9 ± 0.9	4.8 ± 0.9

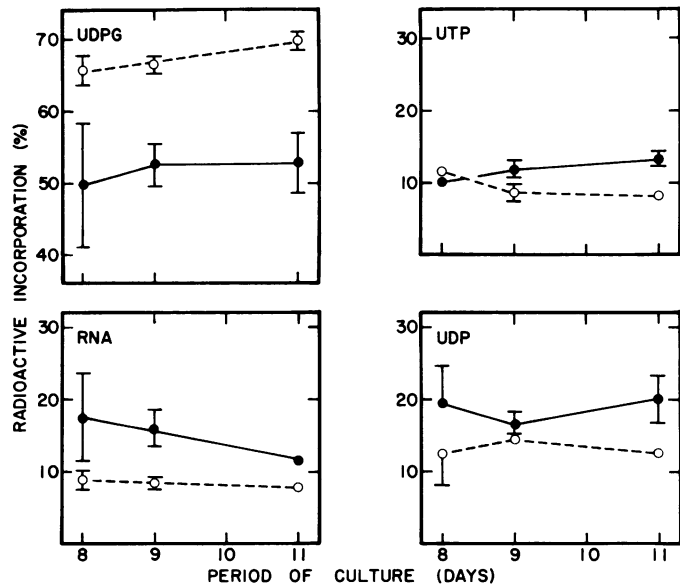[a] Incorporation for 4 h on the 11th day of culture; three to five flasks/treatment.

FIG. 3. Per cent incorporation of [6-¹⁴C]OA into RNA and some pyrimidine pathway intermediates from the 8th to the 11th day of culture, with the medium containing (O– – –O) 100 μM boron or (●———●) 10 μM boron. Each point is the mean of three flasks, and the bars represent 1 SE each side of the mean. Incubation was for 4 h with 0.5 μCi-labeled OA/flask (medium reduced to 20 ml/flask before addition of OA). In this experiment, fiber length measurements showed that there was no visible boron-deficiency effect on the fibers, yet an effect was found on the incorporation patterns.

FIG. 2. Autoradiogram of chromatographic separation of labeled compounds in the acid-soluble fraction. Ovules grown in 100 μM boron were incubated for 5.75 h with culture medium containing 5.4 μCi [6-¹⁴C]OA. The acid-soluble fraction from the fibers was extracted with ether, freeze-dried, and the powder dissolved in 0.2 ml water. A 40-μl sample was applied at the origin (approximately 160,000 dpm). The x-ray film was placed on the chromatography sheet for 7 weeks.

of the year. Nevertheless, there were two consistent trends in all experiments: (a) RNA was significantly higher under boron deficiency in all experiments (standard t test or Mann-Whitney U test, at the 5% level). (b) Incorporation into UDP-glucose was significantly lower under boron deficiency. The catabolic pathway compounds had significantly higher incorporation at the low boron concentration compared to controls.

Another experiment showed that boron deficiency effects were manifested differently at different stages of culture (Fig. 3). The incorporation into UDP-glucose in boron-deficient fibers was statistically lower than in the controls. Incorporation into RNA was higher than in controls but decreasingly so until the 11th day of culture. Incorporation into UTP changed from being significantly lower than controls on the 8th day to higher on the 9th and 11th days of culture. The data for UDP were not significantly different from controls.

DISCUSSION

Three major effects of boron deficiency based on OA incorporation have been found in cotton fiber. The first was an inhibition

of total incorporation, an inhibition which was reflected in depressed levels of all the pools examined (Table I). This indicates that either an inhibition of OA uptake occurs at the membrane level, or an initial step in incorporation into the metabolic pools is affected (for example, inhibition of OMP decarboxylase). Both explanations can be substantiated by recent reports. The work of Loughman's group (16) indicated that ion transport is severely affected by boron deficiency. On the other hand, Birnbaum et al. (3) showed that 6-azauracil, an inhibitor of OMP decarboxylase, produces symptoms like those of boron deficiency in cultured cotton ovules, and that both effects could be partially relieved by the addition of uracil which bypasses this reaction.

In the cultured cotton ovule system, there is a complication in that the ovules float on the medium. In this case transport of OA would proceed mostly via the ovule body to the fiber. We have evidence that the inhibitory effect of insufficient boron applies as much to the ovule body as it does to the fiber (unpublished data). Thus, if membrane transport is imparied during boron deficiency, it might affect uptake into the ovule body and/or transport throughout the tissues.

The second major effect of boron deficiency in this system was the enhanced per cent incorporation into RNA. This has been described as an early effect of boron deficiency (6, 8) and is confirmed by our findings. Our data indicate that at 8 or 9 days of ovule culture in 10 μM boron, we were looking at a primary event, or one close to the primary event, of boron deficiency. Further, where we examined RNA per cent incorporation over a culture period from the 8th to the 11th days, there was decreasing RNA per cent incorporation until, at the 11th day, this value was only slightly higher than that of the controls (Fig. 3). Perhaps this is the transition from the "early effect" of increased incorporation into RNA to the "late effect" of a decreased RNA pool as reported in the literature. Assays of RNA in fiber at the 9th day showed that there was a slightly lower pool size in the 10 μM boron fibers compared to controls (statistically significant at the 0.1% level). The controls had a mean of 6.97 A units and the experimentals a mean of 6.08 A units at 660 nm wavelength by the orcinol method (24).

The third major effect of boron deficiency in this system was the lower UDP-glucose per cent incorporation. This confirms the findings of Teare (27) for P. vulgaris roots and supports the conclusion made earlier (9) in in vitro experiemnts, that UDP-glucose pyrophosphorylase activity is decreased under boron deficiency. Dugger and Palmer (10) found that incorporation of labeled UDP-glucose into cell wall material of cotton fibers is also reduced under boron deficiency. There is evidence that UDP-glucose pyrophosphorylase has strong product inhibition (11, 12). Therefore, if UDP-glucose were to accumulate due to nonutilization in synthesis of cell wall material, one would expect rapid inhibition of UDP-glucose pyrophosphorylase. This would result in the observed decreased radioactive OA incorporation into UDP-glucose.

The problem with expressing data in terms of percentages, is that any perturbation in one compound has to be compensated for by the rise or fall in another. An analysis of the present data comparing them with what would be expected if only one compound were affected, indicated that changes in per cent incorporation into UDP-glucose and RNA were independent phenomena.

In summary, the data indicate that boron deficiency in cotton fiber causes a general inhibition of OA incorporation, while at the same time the hypothesis of Birnbaum et al. (3) is confirmed: pyrimidine synthesis intermediates are shunted away from UDP-glucose synthesis and preferentially into RNA synthesis. This could be related directly to cessation of fiber growth due to inhibition of cell wall synthesis.

There was a difference in timing of the boron deficiency effects on [^{14}C]OA incorporation. The general inhibition of incorporation was greater on the 9th and 11th days of culture than on the 8th, whereas the RNA/UDP-glucose effect was already maximal on the 8th day of culture.

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