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# Osmoregulation in Cotton Fiber

## ACCUMULATION OF POTASSIUM AND MALATE DURING GROWTH[1]

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### ABSTRACT

**Kinetics and osmoregulation of cotton (*Gossypium hirsutum* L.) fiber growth (primarily extension) have been studied. Growth is dependent on turgor pressure in the fiber. It is inhibited when a decrease in the water potential of the culture medium due to an addition of Carbowax 6000, equals the turgor pressure of the fiber. Potassium and malate accumulate in the fiber and reach peak levels when the growth rate is highest. Maximum concentrations of potassium and malate reached in the fiber can account for over 50% of the osmotic potential of the fiber. As growth slows down, levels of potassium and malate decrease and turgor pressure declines. Cotton ovules are capable of fixing $H^{14}CO_3^-$ in the dark, predominantly into malate. Fiber growth is inhibited by the absence of potassium and/or atmospheric $CO_2$. We suggest that potassium and malate act as osmoregulatory solutes and that malate, at least in part, arises from dark $CO_2$ fixation reactions.**

Cotton fibers grow as extensions of single epidermal cells on the outer integument of the ovule. Fiber production from unfertilized cotton ovules cultured *in vitro* has been reported from this laboratory (4). Since a positive turgor pressure is considered essential during cell expansion (8), it is reasonable to assume that the growth of cotton fibers should be subject to osmoregulation mediated by the accumulation of solutes. The present studies were undertaken to determine if a positive turgor is essential for the expansion of cotton fibers and if accumulation of solutes is correlated with rate of fiber growth.

We have assumed during the design of these studies that major, effective osmotic agents are potassium and malate, the latter synthesized primarily from nonautotrophic fixation of $CO_2$. Recently, much evidence has appeared that potassium malate is involved in osmotic and turgor responses of plant cells (1, 6, 11, 14, 15). Raw cotton fiber is known to contain substantial quantities of organic acids with malate being the most predominant (13), and salts of malic acid are known to promote the growth of cotton embryos cultured *in vitro* (12).

We present data showing that levels of potassium and malate in the cotton fiber rise during rapid extension growth and fall as the rate of elongation diminishes, all consistent with the above hypothesis for turgor-driven growth (8).

### MATERIALS AND METHODS

Methods for glasshouse production of *Gossypium hirsutum* L. (Acala, SJ-1) and culture of ovules have been described (2). Unfertilized ovules excised from ovaries within a few hours after anthesis were used in all the *in vitro* studies reported herein. Composition of the basal medium was as previously reported (3), except that in the present studies, glucose was the sole sugar supplied. Also, in these studies, the culture medium contained 5 $\mu$M IAA and 0.5 $\mu$M $GA_3$. Each 125-ml Erlenmeyer flask contained 50 ml of medium and all (about 30) ovules from a single ovary (boll). Cultures were maintained at 34 C in the dark. When, for comparison, experiments were conducted on fiber grown *in vivo*, flowers were tagged on the day of anthesis (designated as day 0) and bolls were harvested and ovules were removed at appropriate ages. For potassium and malate determinations, fibers removed from ovules were used.

**Measurement of Fiber Production.** Fiber production was measured in terms of TFU[3] by a stain-destain method previously described in detail (5). One TFU is equal to one absorbance unit of the stain adsorbed to fibers on a set of 20 ovules (each set derived from a single ovary).

Fiber length was determined with calipers, measuring from the ovule epidermis to the tips of fibers produced on the chalazal end of the ovule. Fiber length was determined for 10 ovules from each ovary.

**Determination of Osmotic and Water Potentials.** An isopiestic thermocouple psychrometer (7), coupled with an automatic recorder and calibrated with NaCl solutions, was used. Intact ovules with their associated fibers, grown *in vitro*, were washed and blotted prior to the determination of water potential ($\psi_w$). Frozen-thawed fiber removed from ovules grown *in vitro* was used for determining osmotic potential ($\psi_\pi$). Turgor pressure ($\psi_p$) was calculated from $\psi_w$ and $\psi_\pi$ assuming the relationship: $\psi_w = \psi_\pi + \psi_p$.

**Determination of Potassium Content.** Fiber samples of known dry weight were digested in 15 ml of hot, concentrated $HNO_3$. When the solid material had completely dissolved, 5 ml of 70% perchloric acid were added. The fiber extract was evaporated to about 2 ml, cooled to room temperature, and diluted to 20 ml with distilled $H_2O$. Potassium content was determined with a Beckman flame spectrophotometer (Model B). A series of KCl concentrations were used as a standard.

**Determination of Malate Content.** Fresh fiber samples of known weight were subjected to methanol-chloroform-$H_2O$ extraction

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[3] Abbreviation: TFU: total fiber units.

and to chromatography on Dowex 50 ($H^+$ form) and Dowex 1 (formate form) ion exchange columns to prepare an organic acid fraction (17). The contents of this organic acid fraction and also standard samples of individual organic acids were made into derivatives by the method of Horning *et al.* (10). The derivatives were then separated and identified by GLC (using a Hewlett-Packard 700 gas chromatograph) on 1.83-m stainless steel columns prepacked with Chromosorb AW coated with 5% OV-17. A temperature program of 2 C/min from 70 C to 210 C was used.

The malate content of the organic acid fraction obtained from the fibers was determined by the enzymic method of Hohorst (9) using porcine heart malic dehydrogenase, NADH, and hydrazine hydrate-glycine buffer. A Beckman DB-G spectrophotometer was used to determine the oxidation of NADH. A standard curve was prepared with authentic L-malate.

**Demonstration of Ability to Incorporate $H^{14}CO_3^-$.** Culture flasks were fitted with serum caps and to each flask (about 30 ovules) 40 $\mu$Ci of $NaH^{14}CO_3$ (55 mCi/mmole) were added. Incorporation was allowed for 1 hr. Ovules were then washed and extracted using methanol-chloroform-$H_2O$ (17). Fractions containing amino acids, organic acids, and sugars were prepared using Dowex 50 ($H^+$ form) and Dowex 1 (formate form) ion exchange columns. Total radioactivity in each fraction was determined by liquid scintillation. The organic acid fraction was subjected to TLC on silica gel, and the acids were detected by autoradiography (17). Radioactivity in each acid was determined by removing the corresponding gel portion and subjecting it to liquid scintillation counting.

**Demonstration of Requirement for $CO_2$.** Culture flasks containing ovules were incubated in a sealed glass vessel which contained a 5 N KOH solution and was connected to the outside atmosphere through an ascarite column. A control set was run without KOH and ascarite. After 14 days, TFU and fresh weight of the ovules plus fiber were determined.

**Demonstration of Requirement for Potassium.** Ovules were cultured in varying concentrations of $K^+$ and TFU were determined after 14 days. Thin tangential slices from chalazal ends of ovules were similarly cultured with and without $K^+$ and were photographed after 14 days.

Potassium was supplied as $KNO_3$ in the normal culture medium. When varying concentrations of $K^+$ were used, the balance of nitrate was supplied as $NaNO_3$. It was first shown that $Na^+$ did not have effects of its own on fiber growth.

## RESULTS

**Fiber Production.** The cumulative increases in TFU and fiber length are shown in Figures 1 and 2 for ovules grown *in vivo* and *in vitro*, respectively. In both cases, there is little growth during the first 4 days, but thereafter growth rate increases. In the case of ovules grown *in vivo*, (Fig. 1) fiber growth slows down after day 17, and a maximum of about 21 TFU are produced. The increase in fiber length shows a similar trend and a final length of more than 2.5 cm is obtained. Results from fibers grown *in vitro* (Fig. 2) show similar trends, except that growth slows down after day 13, and maximum TFU and fiber length obtained are considerably less than those for fibers produced *in vivo*.

**Water Relations of Fiber.** Values of $\psi_\pi$, $\psi_w$, and $\psi_p$ are shown in Figure 3, for fiber grown *in vitro*. The osmotic potential ($\psi_\pi$) remains at about $-7$ bars from day 9 to day 13. Thereafter it declines and is maintained at about $-5$ bars after day 17. Water potential ($\psi_w$) shows little change and is maintained at $-4$ to $-5$ bars. The calculations show that turgor pressure ($\psi_p$) is maintained at about 2.5 bars from day 9 to 13 and declines steadily thereafter to a minimum by day 17.

**Turgor Requirement for Growth.** Length of fiber and TFU

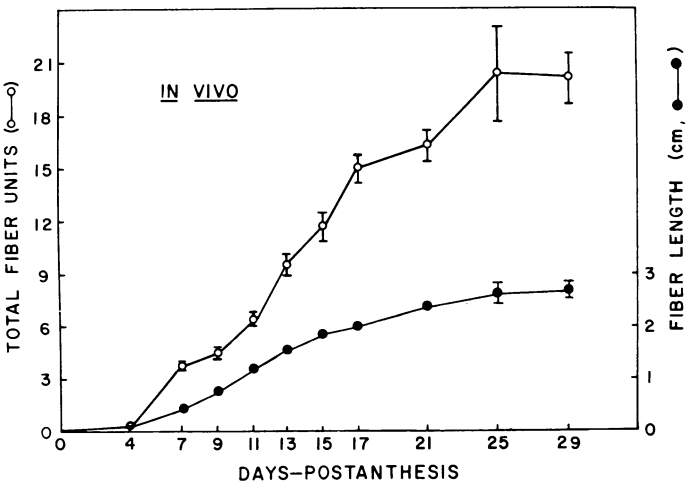


FIG. 1. Cumulative increase in TFU and length of fiber grown *in vivo*. Each value for TFU and length is a mean of 8 and 80 replicates, respectively. Vertical bars represent two standard error values above and two below the means.

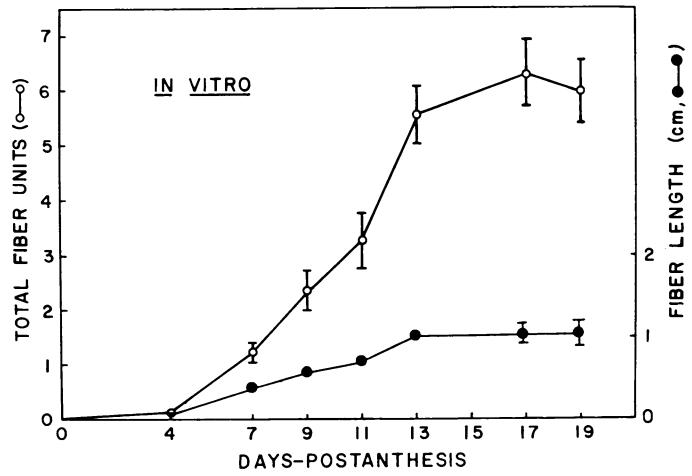


FIG. 2. Cumulative increase in TFU and length of fiber grown *in vitro*. Each value for TFU and length is a mean of 8 and 80 replicates, respectively. Vertical bars represent two standard error values above and two below the means.

produced by ovules cultured in the presence of increasing concentration of Carbowax 6000 are shown in Figure 4. Both fiber production and length are sharply decreased by the addition of as little as $-3$ bars of Carbowax 6000 to the culture medium. Nearly complete reduction of TFU is obtained at $-5$ bars of Carbowax 6000.

**Potassium and Malate Contents.** Gas-liquid chromatographic determination of organic acids showed only malate and citrate in detectable amounts, malate being several times more abundant than citrate (data not shown). Subsequent quantitative estimates of malate were made enzymatically.

Levels of $K^+$ and malate at successive growth stages are shown in Figures 5 and 6 for fibers grown *in vivo* and *in vitro*, respectively. Levels of $K^+$ and malate increase rapidly and reach maxima at day 15 during *in vivo* (Fig. 5) and at day 12 during *in vitro* (Fig. 6) growth; thereafter, levels decline. The levels of $K^+$ and malate are considerably higher in fibers grown *in vitro* than those grown *in vivo*.

**Concentration of Potassium and Malate in Fiber.** From the observed values for $K^+$, malate, and water contents of the fiber

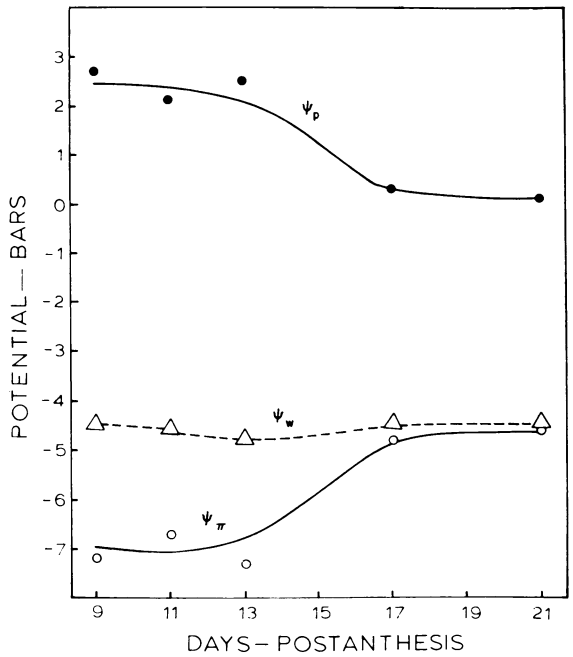


FIG. 3. Osmotic potential ($\psi_\pi$), water potential ($\psi_w$), and turgor pressure ($\psi_p$) of *in vitro*-cultured ovules at different growth stages. Each value is a mean of two replicates.

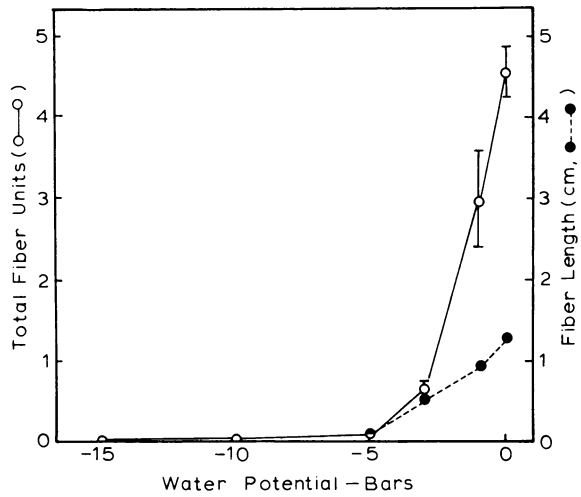


FIG. 4. Inhibition of TFU and fiber length by decrease in $\psi_w$ of the culture medium due to the addition of Carbowax 6000. Each value is a mean of six replicates. Vertical bars indicate two standard error values above and two below the means. Values of $\psi_w$ on the abscissa are due to added Carbowax 6000 alone and do not include the $\psi_w$ of the culture medium (about −5 bars).

grown *in vitro*, approximate concentrations of $K^+$ and malate were calculated and are shown in Figure 7. From day 8 to day 12, concentrations of $K^+$ and malate increase from approximately 68 mM to 85 mM and from 45 mM to 60 mM, respectively. Thereafter, concentrations decline for both solutes.

**Incorporation of $H^{14}CO_3^-$.** Of the total radioactivity incorporated, 80% was in anionic, 18% in cationic, and 2% in the neutral fraction. Of the 80% radioactivity in the anionic portion, about 65% was in malate, 10% in citrate, and 5% in other unidentified organic acids.

**Atmospheric $CO_2$ and Fiber Growth.** Fiber production from ovules cultured in the presence or absence of $CO_2$ is shown in Table I. Fresh weights of ovules plus fiber are also shown in Table I. Fiber production is reduced by nearly 50% in the absence of $CO_2$. On the contrary, fresh weight of ovules plus fiber nearly triples in the absence of $CO_2$, primarily because of ovule callus growth. When the TFU are expressed on the basis of unit

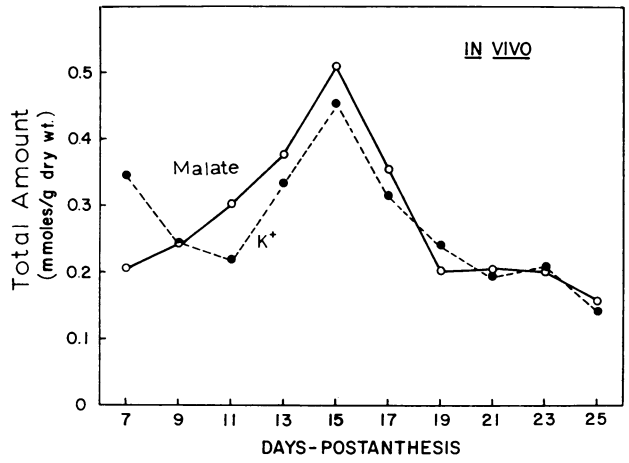


FIG. 5. Potassium and malate levels in *in vivo*-grown fiber at different growth stages; each value is a mean of two replicates.

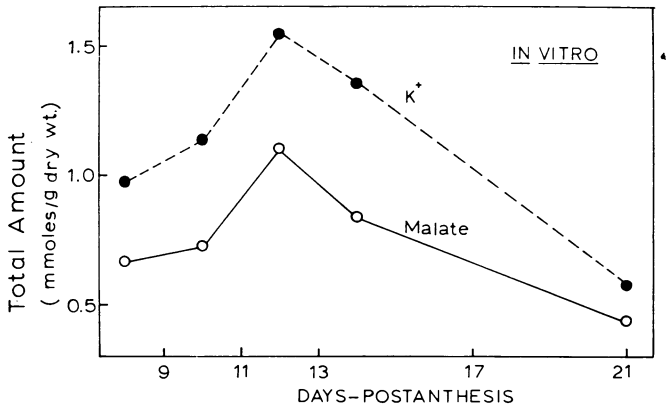


FIG. 6. Potassium and malate levels at different growth stages in fibers grown *in vitro*. Each value is a mean of two replicates.

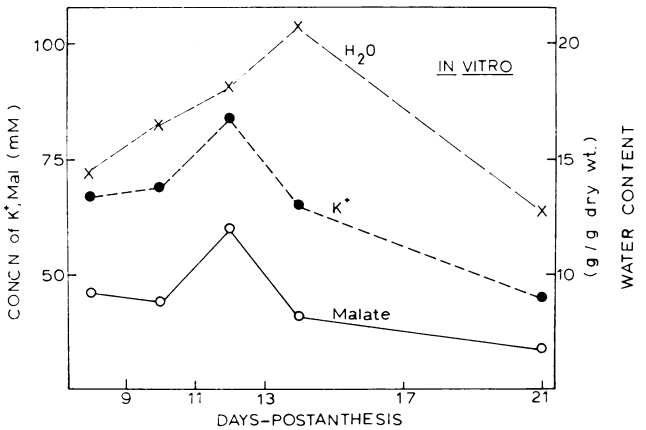


FIG. 7. Potassium and malate concentrations in the fiber at different growth stages *in vitro*. Water content of the fiber is also shown. Values for concentration were derived from absolute amounts of $K^+$ and malate and water content. Each value is a mean of two replicates.

fresh weight, reduction of TFU brought about by the lack of $CO_2$ becomes more pronounced (about 85%).

**Potassium and Fiber Growth.** Fiber production from ovules cultured in various concentrations of $K^+$ is shown in Figure 8. Concentrations up to 0.05 mM do not support measurable fiber growth. With higher concentrations, growth increases rapidly achieving maximum TFU at 10 mM $K^+$.

Tangential slices from chalazal ends of ovules, cultured in the absence of $K^+$, increase in size slightly but do not show fiber growth beyond small protuberances (Fig. 9).

Table I. *$CO_2$ Requirement for Fiber Growth*

Ovules were transferred to culture medium on day 0 in the presence ($+CO_2$) or absence ($-CO_2$) of atmospheric $CO_2$ and after 14 days of growth, TFU, fresh weight of ovules plus fibers, and pH of the culture medium were determined.

| Treatment | TFU | Fresh Wt | TFU/g Fresh Wt | Terminal pH of Culture Medium |
|---|---|---|---|---|
| | | g | | |
| $+CO_2$ | 5.44 ± 0.29 | 0.536 | 10.013 | 5.25 |
| $-CO_2$ | 2.85 ± 0.25 | 1.750 | 1.63 | 5.20 |

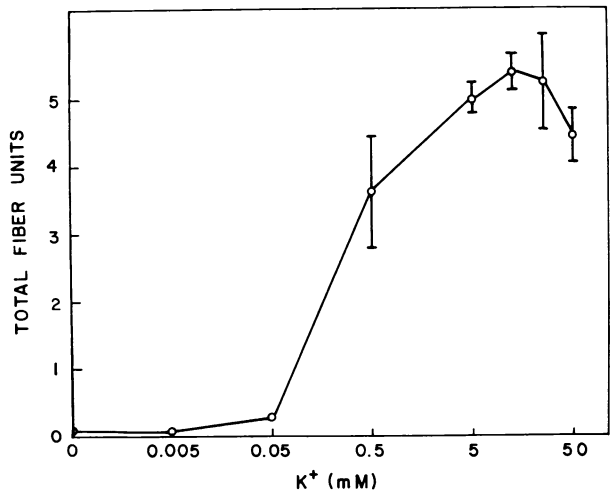


FIG. 8. Effect of $K^+$ concentration on TFU. Each value is a mean of six replicates. Vertical bars indicate two standard error values above and two below the means.

## DISCUSSION

These studies show that (a) extension of cotton fiber is dependent upon turgor pressure; (b) levels of $K^+$ and malate in the fiber show rise and fall in correlation with growth rate; and (c) fiber growth is adversely affected by the absence of both atmospheric $CO_2$ and $K^+$.

The significance of turgor during cell extension is widely recognized and has been reviewed recently by Cleland (8). Our results show that growth of cotton fiber is sensitive to a decrease in turgor of the fiber. The correlation between calculated values of turgor pressure during rapid growth (2–3 bars) and concentration of Carbowax 6000 needed to cause almost complete inhibition of growth (−3 bars) is good. Also, as growth stops, turgor pressure in the fiber falls to less than 0.5 bars.

Our studies also demonstrate that during growth of cotton fiber, both *in vivo* and *in vitro*, $K^+$ and malate levels fluctuate in correlation with growth rate, reaching a peak during the time when growth rate is maximum. The parallel behavior of $K^+$ and malate also suggests their interrelationship as counterions. The combined concentrations (0.15 M) of $K^+$ and malate during the period of highest growth rate can account for more than 55% of the total osmotic potential of the fiber. It should also be noted that the fluctuating component of the osmotic potential, equal to the turgor pressure, can be entirely accounted for by $K^+$ and malate concentrations. The contribution by other solutes to the osmotic potential is not excluded. It is likely that while the basal level of osmotic potential is maintained by all the solutes present in the fiber, the fluctuating component of the osmotic potential is maintained largely by $K^+$ and malate. There are several reports in the literature demonstrating the involvement of these two solutes in other turgor-dependent phenomena (1, 6, 11, 14, 15). Our present studies suggest that $K^+$ and malate act as major osmoregulatory solutes during the growth of cotton fiber.

The fate of $K^+$ and malate during the decline in their levels has not yet been determined. While $K^+$ can be transported out of

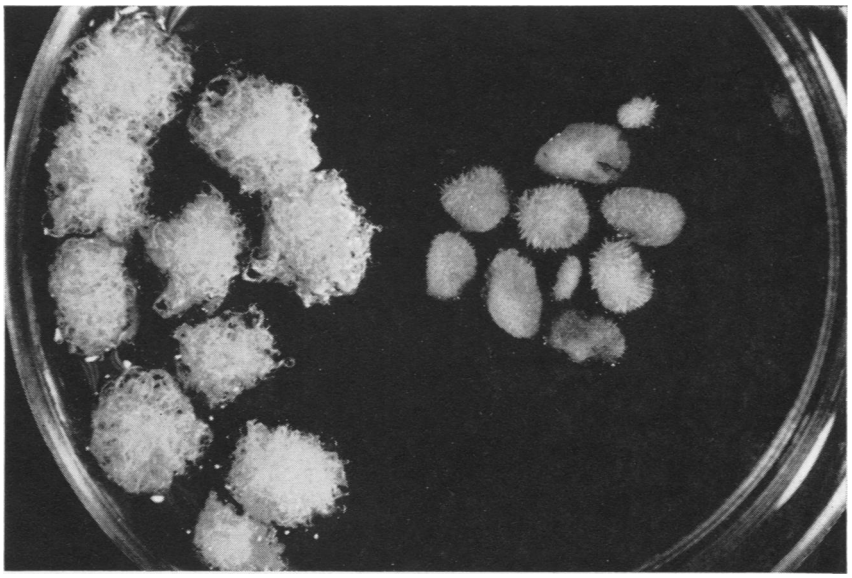

FIG. 9. Production of fiber by thin tangential slices from chalazal ends of ovules, cultured in the presence (left) and absence (right) of $K^+$.

the fiber, we suspect that malate is in a constant turnover pool controlled by the following reactions (16).

$$\text{Phosphoenolpyruvate} \xrightarrow{k_1} \text{malate} \xrightarrow{k_2} \text{pyruvate}$$

The amount of malate in the fiber at any time is determined by the ratio $k_1/k_2$.

Potassium and malate appear to be specifically required for fiber growth. Our results show that ovules do not produce fibers if cultured in medium containing less than 0.50 mM $K^+$ (Fig. 8). Ovules cultured in medium containing less than 0.05 mM $K^+$ show browning and collapse of inner integuments and embryo sac (data not presented). The increase in size, but failure to produce fiber, of chalazal slices cultured in the absence of $K^+$ is noteworthy (Fig. 9). Unlike whole ovules, these slices did not shrivel and die. A malate requirement for fiber growth is also suggested by the present results. We assume that cytoplasmic malate is synthesized from P-enolpyruvate via carboxylation. The ability of ovules to incorporate $H^{14}CO_3^-$ predominantly into malate and the inhibition of TFU by the absence of $CO_2$ (Table I) suggest a requirement of malate for fiber growth. Furthermore, analysis of organic acids from the fiber by GLC showed only malate and citrate, malate being several times more abundant than citrate. An earlier report of McCall and Guthrie (13) also indicated malate and citrate as the predominant organic acids in cotton fiber. Effects of $CO_2$ other than as a substrate for carboxylation are, of course, not excluded. The fact that the ovules produce large amounts of callus in the absence of atmospheric $CO_2$ is not understood. It may be pertinent to point out that abscisic acid, which inhibits TFU and the activities of enzymes, especially of those involved in malate synthesis, also causes production of callus when used in high concentrations (our unpublished results). It is possible that callus formation may be an alternative growth pattern under conditions where fiber growth is inhibited at a biochemical level (*e.g.* carboxylation of P-enolpyruvate).

In summary, our data suggest that potassium malate could act as an important osmoticum for the turgor driven extension growth of cotton fiber and that the malate, at least in part, arises from dark $CO_2$ fixation reactions.

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