Citrate-Induced Citrate Production and Light-Induced Growth of *Blastocladiella emersonii*

By E. C. CANTINO AND AIDA GOLDSTEIN

*Department of Botany and Plant Pathology, Michigan State University,
East Lansing, U.S.A.*

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SUMMARY

Exogenous citrate induced the growing thin-walled ordinary colourless form (OC cell) of the water fungus *Blastocladiella emersonii* to produce more citric acid and, simultaneously, to re-utilize lactic acid liberated previously in one large wave of activity. In the absence of added citrate, release of citric acid was not detectable at the end of the cell's generation time, while production and re-utilization of lactic acid did occur, but in very small amounts and in a series of successive waves. An increase in the generation time of an OC cell, previously known to be induced by visible light, was also induced by exogenous citrate in the dark, provided environmental conditions were favourable. Furthermore, increasing amounts of exogenous CO₂ increasingly decreased the dark generation time; this inhibition was annulled by light. Some effects of gaseous (CO₂/O₂) and ionic (Na⁺/K⁺) balance are also described.

INTRODUCTION

Light-stimulated growth of the ordinary colourless organism (OC cell) of *Blastocladiella emersonii* was first detected with multiple generation cultures in peptone + yeast-extract + glucose (PYG) medium (Cantino & Horenstein, 1956). It was later shown (Cantino & Horenstein, 1959) with synchronized single-generation cultures (SSGC) grown on PYG agar media, whereon it was in part reflected in a bicarbonate and/or CO₂ dependent prolongation of the cell's generation time (GT). Finally, both accelerated growth and the GT effect were achieved (Goldstein & Cantino, 1962) with SSGC in liquid Difco PYG medium supplemented with 5×10^{-3} M-phosphate (pH 6.7; PYG-P). However, the liquid medium which yielded the best synchronized cultures, i.e. in which uniform cell suspensions were maintained most consistently with the least effort, was one designed for *B. britannica* (Horenstein & Cantino, 1964) and modified for use with *B. emersonii*: namely a Difco PYG medium made up in 1.1×10^{-2} M-Na₂HPO₄ and 3×10^{-3} M-citric acid (pH 6.6; PYG-PC). But in spite of its advantages, we had difficulty in demonstrating light-induced prolongations of *B. emersonii* generation time on this medium; the light effects were slight at best and not always reproducible. The purpose of the present report is to show that there was no light effect in PYG-PC medium because citrate substituted for visible light as an inducer of the extended generation time, although modification of the gaseous and ionic balance did permit it to occur even in the presence of citrate. Pertinent terminology, background history, and cultural procedures applicable to this water

fungus are covered in reviews (Cantino & Lovett, 1964; Cantino, 1966; Lovett, 1967); past studies of the effects of light on *B. emersonii* were summarized recently (Cantino, 1965).

METHODS

All synchronized single-generation cultures of OC cells of *Blastocladiella emersonii* were started with spores and propagated in white light (500 ft.c.) and darkness, at 24°, according to Goldstein & Cantino (1962) in either peptone + yeast-extract + phosphate medium (PYG-P) or in a similar medium + citrate (PYG-PC), but with numerous modifications as described below. Citric acid was determined by Stern's (1957) method; lactic acid, according to Ryan (1958).

RESULTS

Comparison of medium without citrate (PYG-P) and with citrate (PYG-PC)

Uninoculated PYG-P and PYG-PC, autoclaved and non-autoclaved, were illuminated (500 and 2000 ft.c.) for 18 hr; ultraviolet (u.v.) and visible absorption spectra were taken thereon. Other samples and untreated controls were chromatographed in several solvent systems; papers were examined under u.v. radiation and developed with ninhydrin, ammoniacal AgNO_3 and other reagents. No significant chemical differences (other than in citric acid) between the two media were detected which were judged to be important factors in, or which provided additional clues about the reason for, the differences in response of *Blastocladiella emersonii* to light in these two media.

Comparison of metabolic products in PYG-P and PYG-PC media

Blastocladiella emersonii was grown in PYG-P and PYG-PC media. Citric acid was not detected in the PYG-P at generation time (GT), while in PYG-PC medium citrate actually increased (Fig. 1); its final concentration in light-grown and dark-grown cultures was about the same. Thus, the presence of exogenous citric acid induced *B. emersonii* to make more of it. Large quantities of lactic acid were also liberated but were re-utilized during the period when citric acid was released (Fig. 2). In PYG-P medium, small amounts of lactic acid were produced and then consumed again, but in at least six successive waves of activity (Fig. 2); by the end of one generation time, little lactate had accumulated.

Effect of aeration rate and O_2/CO_2 balance

Some fifty experiments, in which the aeration rates varied between 1600 and 4200 ml./min., led us to conclude that light-induced increases in generation time were small or negligible at low flow rates (i.e. 1500 ml./min.) but greater at higher flow rates (i.e. 3400 ml./min.; for example, see controls, shaded vs. unshaded bars, Fig. 3). At this higher aeration rate cultures were grown under elevated O_2 tensions. The results showed that increased O_2 (a), did not affect the generation time of dark grown cells (Fig. 3, controls, shaded, vs. 8% O_2 , shaded); (b), at best, slightly augmented the effect of light on the generation time; (c), combined with the removal of CO_2 from the air stream, induced an increase in the dark generation time; i.e. the generation time was maximum and about the same for both light-grown and dark-grown organisms.

Effect of Na⁺ concentration

The generation time was affected by exogenous Na⁺ (Fig. 4); increasing exogenous Na⁺ from 1.6×10^{-2} M to 2.2×10^{-2} M prolonged the generation time by about 4 hr. Below this range, progressive decreases in Na⁺ concentration caused organisms to adhere to the glass vessels; above this range toxicity symptoms appeared.

Fig. 1

Fig. 2

Fig. 1. Accumulation of citric acid in a SSGC of OC cells in PYG-PC, pH 6.6. Aeration, 3600 ml./min.; vol., 600 ml.; population density, 3.1×10^5 /ml. Each point is an average derived from analyses of light- and dark-grown cultures in duplicate.

Fig. 2. Production and re-utilization of lactic acid in SSGC of OC cells in PYG-P and PYG-PC, both pH 6.6. Aeration, 3600 ml./min.; vol. 600 ml.; population density, 3.1×10^5 /ml. for PYG-PC and 2.3×10^5 /ml. for PYG-P; open circles, light-grown; shaded circles, dark-grown. Similar results for the lactic acid content of spent PYG-PC were published previously (Cantino, 1965); new and more extensive data are shown here for comparison with the analyses of PYG-P.

Effect of cationic balance

The generation time was also affected by exogenous K⁺. For example, with phosphate buffer provided as a mixture of Na and K salts, 8×10^{-3} M for each cation, light induced increases of generation time of 120 min and more. However, the OC cells did not discharge their spores. This effect was obtained when Na⁺:K⁺ ratios reached unity, and often with ratio value up to 3; in such cultures, the final pH values were no different from the controls containing only Na⁺. Replacement of all the Na⁺ with K⁺ (i.e. 1.6×10^{-2} M) yielded OC cells which were highly vacuolate and grew poorly; they

were still very small and did not discharge spores even after 28 hr (roughly double the normal generation time). Replacement of Na^+ with NH_4^+ yielded spherical organisms which grew at more nearly normal rates; but again, these were highly vacuolate and not capable of spore discharge after 28 hr.

Effect of CO_2 tension

Blastocladiella emersonii was grown in PYG-PC medium, and the CO_2 in the air stream was varied between 0.03% and 27% (v/v). At one generation time, cell shapes were judged subjectively and estimated quantitatively in terms of the ratio cell width: cell length, and cell volumes calculated therefrom. Differences in generation time between light-grown and dark-grown cultures were recorded. Mature OC cells grown

Fig. 3

Fig. 4

Fig. 3. Effects of O_2/CO_2 balance on the GT and the light-induced increases in GT in SSGC of OC cells in PYG-PC, pH 6.6, using reduced levels of Na_2HPO_4 (0.8×10^{-3} M; i.e. Na^+ , 1.6×10^{-2} M) and citric acid (2.2×10^{-3} M). Each bar represents a separate 600 ml. culture. Aeration, 3400 ml./min.; population densities varied between 1.1 and 1.5×10^5 /ml. in the different experiments; unshaded, light-grown; shaded, dark-grown.

Fig. 4. Effect of exogenous Na^+ concentration on the GT in SSGC of OC cells in 600 ml. PYG-PC, pH 6.5–6.6. Each point represents a separate culture. Open circles, light-grown; shaded circles, dark-grown. The Na^+ level was varied by adjusting the concentration of Na_2HPO_4 from 0.8×10^{-3} to 1.35×10^{-2} M, and citrate from 2.2×10^{-3} to 3×10^{-3} M. Separate control experiments established that the changes in concentrations of phosphate and citrate were not responsible for alterations in GT. Variations in GT at 1.6×10^{-2} and 2.2×10^{-2} M Na^+ (two main clusters of points on graph) were due mainly to the four different flow rates of the aeration stream (1500, 2400, 2800, and 3400 ml./min.) and the two population densities (1.33×10^5 and 2.6×10^5 cells/ml.) used in these experiments. *Additional note:* a serious bottleneck which prevents taking full advantage of a SSGC is the tenacity with which OC cells can sometimes adhere to the glass wall of a culture vessel. Very many experiments, all involving full scale cultures, were done to identify and thereby to circumvent some of the causes involved. These included treatment of glass surface with various agents, and alterations (in various combinations) of pH, aeration rates, spore population densities, and concentrations of HCO_3^- , total phosphate, HPO_4^{2-} , total citrate, citrate $^{3-}$, Na^+ and K^+ . We concluded (a) that the stickiness of cells was seldom if ever due to incorrect concentrations of HPO_4^{2-} , HCO_3^- , or citrate $^{3-}$, to pH *per se*, or to an incorrect balance between total phosphate and total citrate, or between Na^+ and K^+ ; and (b), that insufficient amounts of total phosphate and/or total Na^+ were most likely responsible.

in the dark under 6.7% and 13% (v/v) CO₂ were elongated and only about 1/3 normal size; this inhibitory effect of CO₂ on volume was overcome by illumination at 6.7% (v/v) CO₂ but not at 13% (Table 1). Increases in CO₂ up to 13% led to roughly proportional light-stimulated increases in generation time over the values for dark controls; however, the generation times of all light-grown organisms was essentially the same. Thus, the capacity of CO₂ to yield light-induced increases in generation time on citrate media was actually due to CO₂-induced decreases in the generation time of dark-grown organisms, these decreases being overcome by illumination.

Table 1. *Effect of % CO₂ in the air stream on the volume, shape, and GT of OC cells grown in light and darkness on PYG-PC*

SSGC volumes were 600 ml.; aeration, 1500 ml./min.; population density, 1.17×10^8 /ml.; initial pH, 6.5–6.6 in all cultures; final pH, 6.5–6.6 for 0.03% and 0.5% CO₂, 6.4–6.5 for 6.7% CO₂, and 6.3–6.4 for 13% CO₂. Dark-grown controls without CO₂, when adjusted to pH 6.3 and 6.4, did not exhibit decreased GT. Attempts to increase the CO₂% in the air stream up to ca. 27% failed due to harmful decreases in pH to 6.0 and below at GT. Slight increases in phosphate buffer failed to prevent the pH from dropping too far. Further increases in Na₂HPO₄ to more than 2.5×10^{-2} M, with or without increases in citric acid to 5×10^{-3} M, caused spores to disintegrate (see Fig. 4 and text section on cationic balance). Note that controls in this sort of experiment are particularly critical; the GT depends in part upon aeration rate, population density, Na⁺ concentration, etc., various combinations of which can lead to a GT as high as 19 hr or as low as 14 hr.

% CO ₂	Light			Dark		
	Volume (μ ³)	Width:length	GT (hr)	Volume (μ ³)	Width:length	GT (hr)
13.0	6.2×10^4	0.62	17.0	5.9×10^4	0.68	15.3
6.7	22.6×10^4	0.50	16.9	6.0×10^4	0.66	15.8
0.5	20.0×10^4	(ca. 0.8)	17.1	19.0×10^4	(ca. 0.8)	16.7
0.03	18.8×10^4	0.82	17.1	18.3×10^4	0.84	16.9

Table 2. *Comparison of GT and volume of OC cells grown in light and darkness in PYG-P and PYG-PC*

Growth on PYG-P: values shown are averages for two experiments, each one involving light- and dark-grown cultures in duplicate; population densities were 0.9×10^8 /ml. and 2.5×10^8 /ml.; all other details were as described by Goldstein & Cantino (1962). Growth on PYG-PC: average values are shown for six different experiments, all cultures in duplicate; population density, 1.3×10^8 /ml.; aeration rate, 1500 ml./min.; initial and final pH, 6.55–6.6 in all instances.

	Medium			
	PYG-P		PYG-PC	
	Light	Dark	Light	Dark
GT (hr)	17.5	16.0	17.2	17.1
Cell volume (μ ³)	19.3×10^4	8.0×10^4	18.2×10^4	18.3×10^4

Lastly, the generation time and the volume at one generation time of organisms grown without CO₂ supplements in light and darkness on PYG-PC medium were compared with organisms similarly grown on PYG-P medium (Table 2). In PYG-P medium, light-induced increases (about 90 min.) in generation time were associated with about twofold light-induced increases in cell volume to about 19×10^4 μ³ (see Goldstein & Cantino, 1962, for details about this effect). In PYG-PC medium, dark-

grown organisms displayed the same generation time and volume as those grown in the light in either PYG-PC or PYG-P media, i.e. with or without added citrate. Thus, given suitable CO_2 and Na^+ concentrations, aeration rates, and population densities, exogenous citrate effectively substituted for white light as an inducer of increased size and an extended generation time of an OC cell.

DISCUSSION

Production and re-utilization of lactate in PYG-P

Although our studies dealt primarily with the growth of *Blastocladiella emersonii* in PYG-PC, production and re-utilization of lactate in PYG-P is noteworthy. Its rhythmic appearance and disappearance in successive waves must reflect some endogenous cyclic process during exponential growth of an OC cell. We suspect that a succession of synchronous nuclear divisions is the cause. The motile spore of this fungus is uninucleate; at maturity, the OC cell derived therefrom can produce a few hundred to perhaps 2000 such spores, depending upon its size at GT. Assuming that a germling becomes binucleate by 3 hr (Turian & Cantino, 1959), that the doubling time for nuclei lies between one and 2 hr, and that nuclear divisions during exponential growth are synchronous, some 8 to 12 replications would suffice to generate the required number of nuclei for the next generation of spores. If metabolism of lactic acid were associated with mitosis, production and re-utilization of lactate in 8 to 12 successive waves might occur; in the present study, 8 waves were detected. But judging from the fact (Turian & Cantino, 1959) that the nuclear doubling time in an OC germling in PYG is *ca.* 2 hr in the dark but more like 1.2 hr in the light, and assuming that such differential rates of nuclear reproduction continue throughout ontogeny, more 'lactic acid waves' should have been seen in light-grown cultures than in dark-grown ones. Although the results (Fig. 2) do not show this, analyses were only made at hourly intervals between 9 hr and GT, and even less frequently during the earlier stages in development. A systematic approach with analyses for lactic acid at much more frequent intervals throughout the ontogeny of light- and dark-grown cells should help determine if this notion has validity.

The relation between citrate-induced and light-induced increases in GT of an OC cell

In 1956 (for all references to *Blastocladiella emersonii* in this discussion, see Cantino, 1965), we found that a mixture of OC cells of various ages, pre-grown in multiple generation culture and then resuspended in $\text{H}^{14}\text{CO}_3^-$, consumed more ^{14}C in the light than in the dark and simultaneously accumulated a greater intracellular pool of succinate- ^{14}C . From this and other work, it was hypothesized that light-stimulated growth of an OC cell, and the light-induced CO_2 -dependent extension of its GT, may have been due to increased reductive carboxylation of α -ketoglutarate to isocitrate and its cleavage to succinate and glyoxylate. Some *in vivo* corroboration came from demonstrations that an equimolar mixture of exogenous succinate and glyoxylate did, in fact, substitute for white light by inducing an extended GT of a dark-grown cell. We have now shown that exogenous citrate can also substitute for light by inducing prolongation of the cell's GT, and that—unlike the $\text{HCO}_3^-/\text{CO}_2$ dependent light induction *per se*— CO_2 is no longer necessary when citrate is provided directly. Assum-

ing that citrate gives rise to isocitrate within the cell, this observation lends new support for our belief that the light-induced extension of the GT of *B. emersonii* stems directly or indirectly from a light-induced acceleration of the sequence: α -keto-glutarate + CO₂ → isocitrate → succinate + glyoxylate.

In previous reports, we discussed possible connexions between this reaction sequence and other demonstrable effects of light upon *Blastocladiella*: effects upon its nuclear reproduction and the need for thymidine; upon its capacity for glycine uptake and the latter's relation to exogenous CO₂, intracellular isocitratase activity, and DNA production; upon its genesis of protein and polysaccharide during growth, etc. Assuming that the *in vivo* effect of citrate and of the combination succinate plus glyoxylate have a common basis, there is no need to cover this ground again for it is discussed fully elsewhere.

However our work with citrate does raise unexpected questions for which no clear-cut answers are available. For example, if citric acid substitutes for light by functioning *in vivo* as the progenitor of succinate and glyoxylate, why does exogenous CO₂ depress the GT in the dark when citrate is present? Does CO₂ perhaps inhibit entry of lactate via pyruvate into the tricarboxylic acid cycle, or affect decarboxylations therein, or accelerate formation of citrulline via synthesis of carbamyl phosphate? A case could be made for all these possibilities (not from comparative metabolic maps but from what has been established about *Blastocladiella*'s physiology). Why does exogenous citrate induce accumulation of lactic acid, while little if any appears in its absence, only to have it re-utilized just before sporogenesis? And why does exogenous citrate bring about release of *additional* citrate at this stage in ontogeny? What is the relation of these things to other events associated, in particular, with the terminal stages of ontogeny when an OC cell approaches the end of its development but remains capable of additional light-stimulated growth; events such as the light-induced depression of its D-glucose-6-phosphate: NADP oxidoreductase; the light-induced change in its intracellular distribution of haemoprotein; the sharp rise in its L-glutamine amidohydrolase, associated with rapid incorporation of glutamic acid into protein but almost no equilibration with its amino acid pool; and its very large increase in L-ornithine carbamoyl transferase, decrease in L-arginine ureohydrolase, and rapid uptake of arginine for protein synthesis? And finally, with Wyatt's (1964) provocative discussion of cations and metabolism in mind—especially K⁺ activated, Na⁺ inhibited pathways from phospho-enol-pyruvate and acetate to the Krebs cycle—are the effects of Na⁺/K⁺ balances on the GT related to cationic effects upon activity of the OC cell's tricarboxylic acid cycle? It is tempting to speculate about the interrelationships which must exist among the reactions known to occur in *Blastocladiella*. We shall, however, control this urge in favour of returning to the bench for further work.

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