Effect of External pH on the Internal pH of *Chlorella saccharophila*[1]

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ABSTRACT

The overall internal pH of the acid-tolerant green alga, *Chlorella saccharophila*, was determined in the light and in the dark by the distribution of 5,5-dimethyl-2-[14C]oxazolidine-2,4-dione ([14C]DMO) or [14C]benzoic acid ([14C]BA) between the cells and the surrounding medium. [14C]DMO was used at external pH of 5.0 to 7.5 while [14C]BA was used in the range pH 3.0 to pH 5.5. Neither compound was metabolized by the algal cells and intracellular binding was minimal. The internal pH of the algae obtained with the two compounds at external pH values of 5.0 and 5.5 were in good agreement. The internal pH of *C. saccharophila* remained relatively constant at pH 7.3 over the external pH range of pH 5.0 to 7.5. Below pH 5.0, however, there was a gradual decrease in the internal pH to 6.4 at an external pH of 3.0. The maintenance of a constant internal pH requires energy and the downward drift of internal pH with a drop in external pH may be a mechanism to conserve energy and allow growth at acid pH.

Microscopic algae generally have pH optima for growth and photosynthesis in the neutral to alkaline pH range. There are some species, however, which can grow and photosynthesize in acid conditions and studies with procaryotes have shown that it is necessary for such acidophilic organisms to maintain a neutral internal pH (17). Our knowledge of the variation in internal pH of photosynthetic microorganisms with changes in external pH is limited to only a few species of green algae and cyanobacteria (7, 12, 13, 16).

The internal pH of microorganisms can be conveniently and accurately measured by determining the distribution of a radioactively-labeled weak acid or weak base between the intracellular space and an external solution of known pH. It is assumed in this method that the uncharged labeled compound passively diffuses into the cell so that at equilibrium the concentrations of the uncharged species inside and outside the cell will be equal. Thus, from the pK of the labeled compound, the external pH, and the measured concentrations of the compound inside and outside the cell, the internal pH can be calculated (5). To accurately measure the equilibrium concentrations of the compound in the cells and the medium, the pH of the medium should be ±1.0 to 1.5 units of the pK of the compound and therefore in media of circumneutral pH the weak acid DMO (pK_a 6.32) has been used. In organisms where comparison studies have been feasible, internal pH values measured directly with

glass electrodes and those derived from [14C]DMO[2] distribution were in reasonable agreement (5, 20).

DMO has been used to determine the internal pH of a number of cyanobacterial (7, 10) and green algal cells, particularly those of *Chlorella* species (3, 4, 15, 21), suspended in media in the pH range of 5.3 to 7.3. It has also been used to determine the internal pH of algae in acidic media as low as pH 3.0 (3, 16), which are external pH values outside the useful range of DMO.

The internal pH of acidophilic bacteria has been determined using [14C]BA, an organic acid with a relatively low pK_a (pK_a = 4.19), as a pH probe (2, 14). In this study we have evaluated the use of [14C]BA to determine the pH_i of the acidophilic green alga *Chlorella saccharophila* at acid pH, and using this compound and [14C]DMO, the effect of pH_e between pH 3.0 and 7.5 on the pH_i of *C. saccharophila* has been investigated.

MATERIALS AND METHODS

Chlorella saccharophila (Krüger) Nadson was obtained as an axenic culture from the University of Texas Culture Collection and grown axenically in aerated cultures in Bold's Basal Medium (19) under continuous light at 20 to 23°C.

Cells were harvested by centrifugation at 9,000g and resuspended in appropriate 50 mM buffers: phthalate at pH 3.0 to 5.0; Mes at pH 5.0 to 6.5; phosphate at pH 6.0 to 7.5; and Tricine at pH 7.5. No effects of buffers on the photosynthetic rate of this alga were detected.

Determination of Intracellular pH. The intracellular pH of *C. saccharophila* was determined in the light and dark by measurement of the partitioning of the weak acids [14C]DMO and [14C]BA between the cells and the incubation medium as described for DMO by Espie and Colman (8).

Cell suspensions (0.5 or 1.0 ml) containing about 30 μg Chl/ml which had been preincubated in the light at a light fluence of approximately 1000 $\mu E \cdot m^{-1} s^{-1}$, or in the dark in buffer of the appropriate pH were allowed to equilibrate with [14C]DMO or [14C]BA (Amersham Corp., Toronto, Canada; specific activity of 50–54 and 58 mCi/mmol; final concentration, 18.45 and 0.98 μM, respectively) for 10 min. Following incubation, triplicate 50-μl samples of cell suspension were removed and placed, as a top layer, in 400-μl Eppendorf microtubes, containing 100 μl of a 6% (w/v) sorbitol solution as a bottom layer and 100 μl of silicone fluid (75% AR 20:25% AR 200; Wacker Chemie, Munich, F. R. G.) as the middle layer. The cells were separated from the incubation medium and collected in the sorbitol phase by centrifugation at 12,500g for 45 s in an Eppendorf microcentrifuge, model 5412. The tubes were then quickly frozen in a dry ice-methanol mixture. The pH of the incubation medium was measured with a Fisher Accumet (model G10) pH meter,

[2] Abbreviations: [14C]DMO, 5,5-dimethyl-2-[14C]oxazolidine-2,4-dione; [14C]BA, [14C]benzoic acid; pH_i, internal pH; pH_e, external pH; RuBP, ribulose-1,5-bisphosphate.

equipped with a microelectrode. This pH value was used in subsequent calculations of intracellular pH.

The total intracellular volume of the cell suspension was estimated by incubating 0.5 ml of the cell suspension with 65 μl of ^3H_2O (111.9 μCi/ml) for 10 to 15 min in the dark. Following incubation, triplicate 50-μl aliquots of suspension were removed, layered on silicon fluid in 400-μl microtubes as described above, and centrifuged for 45 s. This resulted in an estimate of the intracellular volume plus extracellular space between the cells. The extracellular space alone was measured as above except that the cells were incubated in 25 μl of [^3H]Inulin (904 mCi/mmol, Amersham). The intracellular volume was then determined by subtracting the extracellular volume from the total cell volume. Typical recoveries of cell Chl which passed through the silicone oil into the sorbitol during centrifugation ranged from 75 to 90% of the total Chl added. Chl was measured spectrophotometrically in 100% methanol according to the method of Holden (11).

To determine the amount of radioactivity associated with the filtered cells, the microtubes were cut at the sorbitol-silicone oil interface while still frozen. Once the sorbitol, containing the cell pellet had melted, it was removed with a pipette, and the tube then washed with approximately 0.3 ml distilled H_2O. The pellets combined with the washings were placed in 5 ml of counting scintillant (ACS Amersham) and counted in a Packard scintillation counter. Averages of triplicate samples were taken to calculate intracellular DMO or BA concentrations and the intracellular pH by the method of Werdan et al. (23) using a pK_a of DMO at 25°C of 6.32 (1) and a pK_a of BA at 25°C of 4.19 (22).

Metabolism and Intracellular Binding of DMO or BA. To ensure that DMO or BA were not metabolized by *C. saccharophila* cells, samples of cells were incubated in the light with each of the probes as described above for 45 min, and the cells washed and then extracted with 95% ethyl alcohol. Extracts of [^{14}C] DMO-incubated cells were chromatographed on Whatman No. 1 paper using isopropanol:ammonia:water (8:1:1, by volume) (18) while extracts containing [^{14}C]BA were chromatographed on silica gel plates using chloroform:cyclohexane:acetic acid (80:20:1, by volume), in both cases against 5-μl aliquots of the authentic ^{14}C-labeled compound. Paper chromatograms were cut into 1-cm-wide strips, silica gel was scraped from the plates in 1-cm-wide sections and placed in 10 ml ACS scintillation fluid, and the radioactivity was determined as described above.

'Wash-out' experiments were performed to test for irreversible binding of either DMO or BA by allowing 2 ml of the cells to incubate in either of the probes for 45 min, spinning them down and washing them three times in appropriate pH buffer at 15-min intervals, and then counting the resulting supernatant and pellet.

Measurement of Photosynthetic Rates. Photosynthetic rates of algal cell suspensions (10–15 μg Ch·ml^{-1}) in buffer at 25°C were measured as O_2 evolution at saturating dissolved inorganic carbon concentrations and a light fluence of 1000 μE·m^{-1}·s^{-1}, in a temperature-controlled Clark-type O_2 electrode calibrated as described previously (8).

RESULTS

Cells of *C. saccharophila* were incubated in the light in medium at pH 4.0 containing [^{14}C]BA. There was a rapid initial uptake of [^{14}C]BA, but after the first 2 min of incubation the amount of ^{14}C taken up remained constant (Fig. 1, top). Similar incubation of cells in medium at pH 7.0 containing [^{14}C]DMO demonstrated a lower rate of ^{14}C uptake, but the amount of ^{14}C taken up became constant after 35 min of incubation. (Fig. 1, bottom).

These results suggest that an equilibration of ^{14}C-labeled BA takes place between the cells and the medium by passive diffusion. To test this, aliquots of cell suspension were incubated for 10 min each with concentrations of [^{14}C]BA ranging from 0.3 to
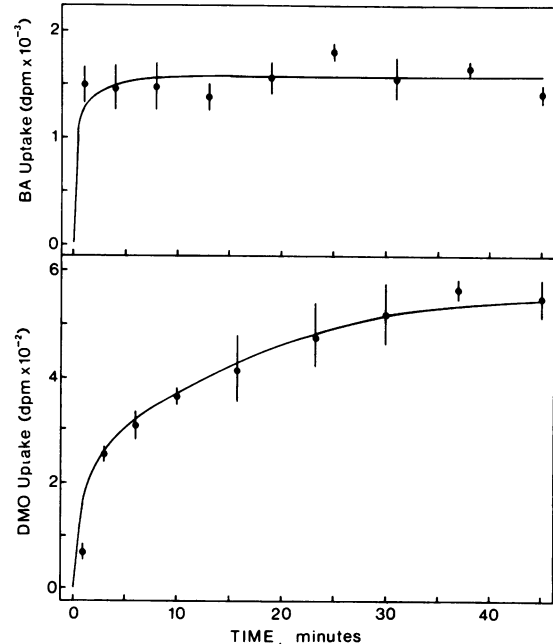

FIG. 1. Time-course of [^{14}C]BA (top) and [^{14}C]DMO (bottom) uptake by *C. saccharophila* cells. Cells were incubated at 25°C, at saturating light fluence, in a medium of pH 4.0 for [^{14}C]BA and pH 6.0 for [^{14}C] DMO uptake. Error bars represent SD from the mean of triplicate samples.
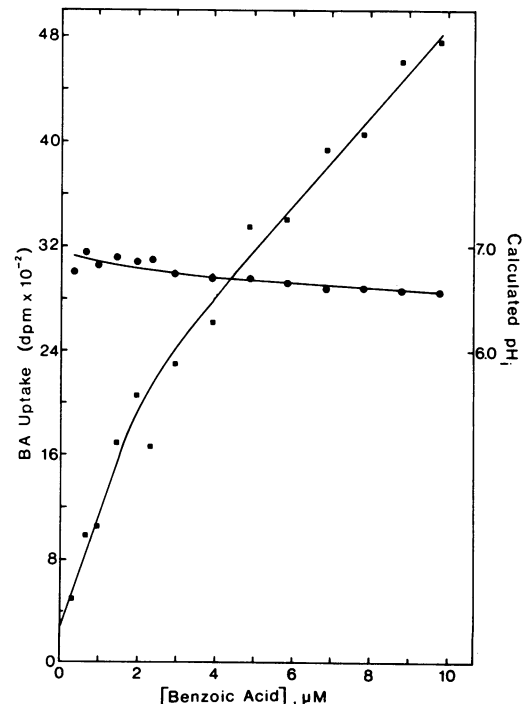

FIG. 2. [^{14}C]BA absorption by *C. saccharophila* cells as a function of [^{14}C]BA concentration ranging from 0.3 to 9.8 μM. Cells were incubated for 10 min at 25°C under saturating light fluence at pH 4.5. Calculated intracellular pH values (●) are also shown.

9.8 μM. The uptake of [^{14}C]BA increased linearly with concentration up to 2 μM, but at higher concentrations this linear relationship was not maintained and the calculated internal pH decreased as the intracellular concentrations of the acid increased (Fig. 2). However, similar incubations of cells for 40 min with [^{14}C]DMO at pH 7.3 and concentrations from 0.5 to 53 μM,
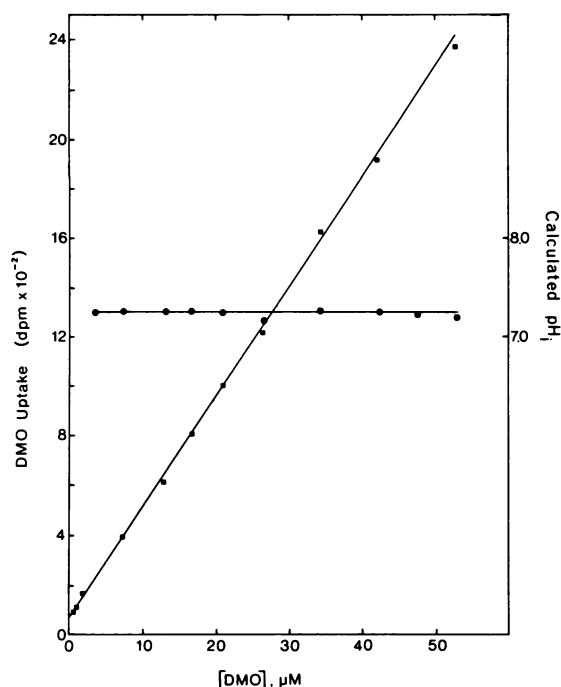
FIG. 3. [14C]DMO absorption by *C. saccharophila* cells as a function of [14C]DMO concentration (■). Cells were incubated at concentrations of [14C]DMO ranging from 0.5 to 53.0 μM for 10 min at 25°C, under saturating light fluence at pH 6.5. Calculated intracellular pH values (●) are also shown.
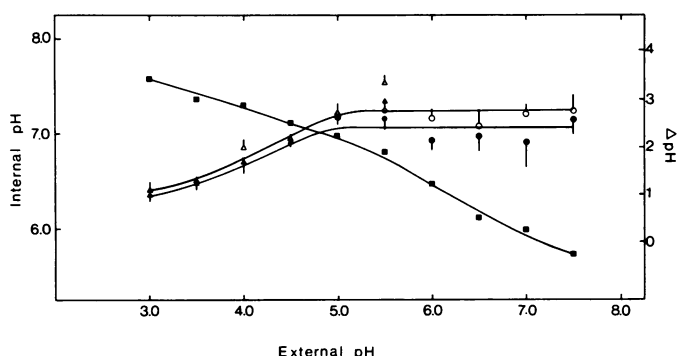

External pH

FIG. 4. Effect of external pH on the intracellular pH of *C. saccharophila* cells measured in light (△, ○) and dark (▲, ●). The intracellular pH was calculated after equilibration of [14C]BA (△, ▲) or [14C]DMO (○, ●) over the external pH range of 3.0 to 7.5. Error bars represent the SE of four to five separate determinations done in triplicate. The pH difference (△ pH) between the external medium and the intracellular pH (■) is also shown.

showed a linear relationship between 14C uptake and DMO concentration (Fig. 3). The calculated internal pH of the cells was constant at pH 7.3 over the entire range of DMO concentrations used (Fig. 3).

Chromatographic analysis of extracts of *C. saccharophila* cells which had been incubated in [14C]BA or [14C]DMO demonstrated that 14C was present only in the supplied compound and no other labeled compounds were detected. This indicates that neither compound is metabolized by these cells.

The [14C]BA and [14C]DMO taken up by cells was readily removed by placing the cells in fresh medium. After allowing cells, preloaded with either [14C]BA or [14C]DMO, to equilibrate three times with fresh medium, less than 1% of the original 14C activity remained. This would indicate that intracellular binding of the compound does not contribute markedly to the uptake of
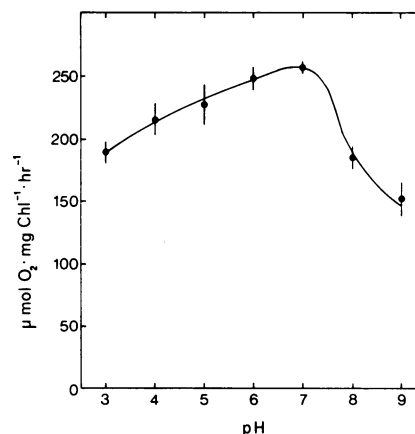

FIG. 5. Effect of external pH on the photosynthetic rate of *C. saccharophila* at 25°C.

either compound.

Effect of External pH on Internal pH. The internal pH of *C. saccharophila* was measured in the light and dark over the external pH range of 3.0 to 7.5, using [14C]BA from pH 3.0 to 5.5 and and [14C]DMO from pH 5.0 to 7.5 (Fig. 4). The intracellular pH remained constant at pH 7.3 in the light and at 7.1 in the dark over the pH$_e$ range of 5.0 to 7.5. Below an external pH of 5.0, the intracellular pH decreased to 6.9 at pH$_e$ of 4.5 down to pH 6.4 at pH$_e$ of 3.0.

At external pH values of 5.0 and 5.5 where the use of the two probes overlaps, there is fairly good agreement between the calculated values of internal pH, suggesting that since the two probes produce comparable results at the same pH, then confidence can be placed on the pH$_i$ values obtained at divergent external pH values.

Effect of pH on Photosynthetic Rate. Photosynthetic activity in *C. saccharophila* was maintained over a wide pH range (Fig. 5). The rate of photosynthesis as measured by O$_2$ evolution exhibited an optimum at pH 7.0 but relatively high rates were maintained as the pH was lowered to pH 4.0 where the photosynthetic rate was 77% of the optimum rate and then dropped to 50% of the optimum rate at pH 3.0. At alkaline pH, photosynthesis was markedly inhibited above pH 8.0 (Fig. 5).

DISCUSSION

The partitioning of [14C]BA between *C. saccharophila* cells and the suspending medium, followed by silicone oil centrifugation has been evaluated as a means of measuring the internal pH of the algal cells. The assumptions upon which this technique is based appear to be valid for the uptake and distribution of benzoic acid: no metabolism of [14C]BA was detected and irreversible binding of the compound was minimal. The uptake of [14C]BA was found to reach equilibrium in less than 2 min at pH 4.0, indicating that the uncharged species was taken up by passive diffusion (Fig. 1). The intracellular concentration of [14C] BA is a linear function of external [14C]BA concentration only over the range of 0.5 to 2.0 μM (Fig. 2) and the calculated internal pH is constant over this concentration range. At concentrations of 2.0 to 10.0 μM, accumulation is not a linear function of concentration as the accumulated acid reduces the internal pH (Fig. 2). These results indicate that at low concentration [14C]BA is a reliable pH probe.

In contrast, the uptake of [14C]DMO was found to reach equilibrium after 35 to 40 min (Fig. 1). Despite this long equilibration time, there was no evidence of metabolism or of irreversible binding of DMO in these algal cells. Even with such slow equilibration the intracellular accumulation of [14C]DMO was found to be a linear function of concentration over a wide

range from 1.0 to 52.5 μM (Fig. 3) which is consistent with the postulate that the compound entered the cell by passive diffusion. Consequently, the internal pH of the cells calculated from [^{14}C] DMO distribution was constant over a wide range of [^{14}C]DMO concentration (Fig. 3).

The useful ranges of the two compounds, BA and DMO, as pH probes overlap in the range pH 5.0 to pH 5.5. The internal pH of C. saccharophila was measured at pH 5.0 and pH 5.5 with both compounds and the results were in good agreement (Fig. 4) further indicating that, at low concentrations, [^{14}C]BA is a reliable pH probe. The values of pH$_i$ obtained with [^{14}C]BA at acid pH$_e$ are certainly less variable than those obtained previously where attempts were made to use DMO as a pH probe at acid pH$_e$ (16).

In the calculation of pH$_i$, the values of the pK$_a$ of both DMO and BA were not corrected for the ionic concentration of the intracellular milieu since this is not known. The intracellular concentration of dissolved ions is certainly high and would cause some suppression of the dissociation of weak acids entering the cell, and thus the calculated pH$_i$ values will be a slight underestimation of the actual value of pH$_i$. However, since there is good agreement between the pH$_i$ values obtained with BA and DMO in the pH$_e$ range pH 5.0 to 5.5, it would appear that the magnitude of the underestimation is the same for both pH probes or that it is less than the experimental errors involved in the determination.

The values of pH$_i$ at acid pH$_e$ obtained in this study using BA as a pH probe (Fig. 4) are somewhat lower than those obtained in another study (3) with the same alga at acid pH$_e$ using DMO as the pH probe. This discrepancy is probably due to the fact that at pH 4.0 a large proportion of the DMO is in the undissociated form which will readily diffuse into the cell. The subsequent large intracellular accumulation of DMO relative to that in the medium may result in the overestimation of the calculated internal pH. When used at low concentration BA does not lower the internal pH of these cells and appears to be a more reliable pH probe than DMO at acid pH.

The overall internal pH of C. saccharophila remains relatively constant at pH 7.3 over a range of pH$_e$ between 5.0 and 7.5. As the pH$_e$ decreases, the ΔpH between the cell and the medium increases and, below pH$_e$ 5.0, the internal pH decreases to pH 6.4 at an external pH of 3.0 (Fig. 4). These values of pH$_i$ represent the overall internal pH of C. saccharophila cells and, thus, in this nonvacuolate cell, they represent the bulk cytoplasmic pH, i.e. the pH of the cytoplasm plus the chloroplast. Since the chloroplast is probably maintained close to pH 8.0 in the light, the actual cytoplasmic pH will be somewhat lower than the overall pH$_i$. At pH$_e$ above pH 5.0, the pH$_i$ is higher in the light than in the dark (Fig. 4). This effect of light on pH$_i$ is similar to that found in leaf mesophyll cells where the chloroplastic pH has been found to be the principal determinant of the cytoplasmic pH (8). At pH$_e$ lower than pH 5.0, however, there is no significant difference between the values of pH$_i$ in the light and the dark (Fig. 4) and this maintenance of pH$_i$ in the dark at a value comparable to that in the light suggests that it may depend on energy derived from respiration.

The gradual decrease in internal pH of C. saccharophila with a decrease in pH$_e$ below pH 5.0 is a finding similar to that suggested by the results of Lane and Burris (16) with an acid-tolerant species of Euglena. Not all photosynthetic microorganisms respond in this way to acid media: in cyanobacteria which are not tolerant to acid pH, the pH$_i$ drops rapidly as pH$_e$ decreases below pH 7.0 (7) or the pH$_i$ remains constant and cell growth is inhibited (13). A similar maintenance of constant pH$_i$ at acid pH also appears to occur in some acid-intolerant green algae (16). The maintenance of internal pH requires energy and the ability to maintain a constant internal pH at an acid external pH would

require an expenditure of energy to pump protons out of the cells (6). The gradual decrease in pH$_i$ found in C. saccharophila at acid pH appears therefore to be a balance between maintaining a circumneutral pH, to allow the metabolic processes of the cell to proceed at an optimum rate, and conserving the energy which would be required to maintain a constant internal pH.

These results indicate that at acid pH$_e$ there is a pH gradient between the cells and the medium (Fig. 4) which would support the intracellular accumulation of inorganic carbon from CO_2 taken up by diffusion in a manner similar to that described for chloroplasts (23) and for higher plant mesophyll cells (9). Thus, at acid pH$_e$, where the bulk of the inorganic carbon is in the form of CO_2, inorganic carbon will accumulate in the cytoplasm in equilibrium concentrations of CO_2 and HCO_3^- determined by the pH$_i$. Active photosynthesis depends upon the maintenance of a saturating concentration of CO_2 at the site of RuBP carboxylase in the chloroplast and the maintenance of such a CO_2 concentration may be mediated by a bicarbonate transport system at the chloroplast membrane as has been suggested by Beardall and Raven (3, 4). The maintenance of a circumneutral cytoplasmic pH will determine the equilibrium HCO_3^- concentration and hence determine the supply of substrate for this transporter.

At pH 7.0, however, the ΔpH is small and at pH 7.5 the pH gradient is reversed, indicating that intracellular inorganic carbon accumulation by diffusion of CO_2 would not take place in this pH range. The optimum pH of photosynthesis in C. saccharophila is pH 7.0 and photosynthesis proceeds actively up to pH 8.0 (Fig. 5). The maintenance of maximum rates of photosynthesis at alkaline pH which limits or precludes the accumulation of inorganic carbon uptake by diffusion of CO_2, suggests that bicarbonate is taken up by these cells to provide an inorganic carbon source for photosynthesis. The mechanism of inorganic carbon uptake into the cytoplasm varies therefore with external pH.

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