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EUGLENA, AS A CELLULAR MODEL USED IN PHARMACOLOGY FOR STUDYING THE EFFECTS OF CITRULLINE MALATE ON LACTATE METABOLIZATION

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Abstract—1. Previous studies have shown that *Euglena* grown in darkness with 33 mM lactate as sole carbon source, metabolized this substrate and accumulated paramylum according to a pathway comparable to the one existing in hepatic cells which transform blood lactate to glycogen.

2. It was possible to use *Euglena* as a cellular model to study the effect of drugs on metabolism and gluconeogenetic synthesis.

3. We have demonstrated that citrulline malate acts as a competitive substrate of lactate, when added at the high concentration of 2 g/l, but that at lower concentration, 0.5 g/l, it provoked a quicker assimilation of the lactate.

4. Using $^{14}CO_2$ as tracer, we detected a reinforcement of carboxylations not accompanied, however, by a labelling of the reserves (paramylum); this suggested that the $^{14}CO_2$ incorporated in intermediary products was quickly degraded.

5. An important augmentation of ATP pool in the presence of the drug could be detected by reference to the cells grown only with lactate, which could be due to the intensification of the respiratory pathway insensitive to cyanide.

6. The addition to the 33 mM lactate culture medium, of 0.5 g/l of citrulline malate induced an acceleration of the lactate consumption correlated with an augmentation of both carboxylations and ATP pool.

7. The generation time was not affected, nevertheless the stationary phase seemed to be attained slightly later in the supplemented cultures.

INTRODUCTION

Euglena is a unicellular protist, which transforms lactate in polysaccharide reserves, the paramylum (β-1-3, glucan), according to a metabolic pathway comparable (Briand *et al.*, 1981) to the one characterizing hepatic cells (Scrutton and Utter, 1968; Di Tullio *et al.*, 1974) which transforms the blood lactate in glycogen. Metabolic processes are almost identical; they consist of a carboxylation of a C-3 molecule, pyruvate in the liver cell and phosphoenol pyruvate (PEP) in *Euglena*, to form oxaloacetic acid (OAA) in the mitochondria. Then follows, in the two cell types, a decarboxylation by a specific enzyme, the PEP carboxykinase guanosine triphosphate (GTP) dependent (EC 4.1.1.149) of the OAA in PEP with a simultaneous transfer of the PEP out of the mitochondria for gluconeogenesis. Synthesis and accumulation of sugar reserves occur on a cytoplasmic promotor in the hepatic cell (Di Tullio *et al.*, 1974) and in a mitochondrial annex in *Euglena gracilis* (Briand and Calvayrac, 1980).

Citrulline malate (Stimol[MD]) is a complex formed by the stoichiometric combination of DL-malic acid and L-citrulline. Clinical assays have shown that this drug displayed antiasthenic properties when taken by convalescent people (Commandré, 1978; Dauverchain, 1982; Creff, 1982; Taillade, 1984; Carbasse, 1985). When administered before an intensive muscular effort, this drug allowed a quicker recovery by speeding up the disappearance of blood lactate (Fornaris *et al.*, 1984).

In the present study, we have used *Euglena gracilis*, organotrophically grown in darkness in a medium containing 33 mM lactate as sole carbon source, in order to study the effect of additional citrulline malate on the lactate assimilation and paramylum storage by the cells. The two tested doses were 0.5 and 2 g/l. We have followed some growth parameters of the cells, the phenomena of carboxylation and decarboxylation, as well as the quantities of cellular ATP and the respiratory criteria.

MATERIALS AND METHODS

The wild strain used in these studies was *Euglena gracilis* Z. Cells were grown, in darkness at 27°C, in a liquid mineral medium supplemented with B1 and B12 vitamins and in lactate (33 mM), the pH was 3.5, and citrulline malate was absent or added. Media were sterilised at 110°C for 30 min. When added, the citrulline malate was filtered through sterile Millipore filters to final concentrations of 0.5 and 2 g/l, before the inoculation of cells (at a cell concentration

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of about 3.10^4 from a preculture 3 days old, i.e. having overrun the exponential phase of growth.

Cell growth

Cell number was determined, after cell immobilisation by IK (final concentration between 5 and 8%), by cell counting on a Malassez cell.

Cell volumes were measured, after cell immobilisation (final IK concentration 5–8%), in a hematocrite apparatus.

Biochemical measurements

Protein determinations were done according to Bradford's technique (Bradford, 1976). Lactate concentrations were followed by the NADH production accompanying the enzymatic transformation of lactate to pyruvate by the lactate dehydrogenase (Kit Boehringer Mannheim, ref. 139084).

Paramylon, extracted from the cells by KOH 30%, was precipitated by absolute ethanol then measured by anthrone (Fales, 1951).

Physiological studies

Carboxylations and decarboxylations. The rates of carboxylation and decarboxylation phenomena were determined by $^{14}CO_2$ incorporation from $NAH^{14}CO_3$. Cell suspensions were sampled at different phases of the growth curves, and concentrated to 10^6 cells/ml, pH being adjusted to 7. The cell sample was transferred to an opaque flask (temperature regulated at 20°C), gently agitated, and swept by a weak CO_2-free air stream. The air was first passed through a washing bottle containing KOH. At time 0, radioactive bicarbonate (specific radioactivity 2.17 GBq/mmol) was injected carrier-free at a final concentration of 38 kBq/ml of cell suspension. The $^{14}CO_2$ arising from bicarbonate is isotopically diluted in the external and intracellular CO_2 pools. The decarboxylated $^{14}CO_2$ was trapped in 10 ml of CO_2-oxifluor (mixture of scintillation solvent and of CO_2 fixative from New England Nuclear).

In order to follow the kinetic of incorporation, aliquots were taken at 0, 15, 30 mn, and at 1, 2, and 3 hr. For each point the quantities of ^{14}C: in the supernatant of the suspension (nonutilized ^{14}C and eventually excreted ^{14}C, which is negligible in Phase I), in the washed cells and in the trapped CO_2, were determined. This was done by transferring 100 μl aliquots of each fraction to 5 ml of Biofluor (New England Nuclear). Radioactivity was determined with a 1211 Rack Beta (L.K.B.) liquid scintillation counter. The initial rates of CO_2 fixation were determined from the tangents to the incorporation curves at time 0; they were expressed as percentage of the total radioactivity supplied to the cells (i.e. ^{14}C in the supernatant, the pellet and the oxifluor). The metabolised $^{14}CO_2$ was considered to be the sum of ^{14}C found into the cells and of the decarboxylated $^{14}CO_2$. All these values were expressed by hr and by 10^6 cells. In addition the paramylum was extracted from the remaining cells, after 3 hr of experiment, and its labelling was measured.

Cell respiration. The respiratory intensity was polarographically determined with a Clark type electrode. For the three types of cultures: 33 mM lactate (control); 33 mM lactate plus citrulline malate (0.5 g/l); 33 mM lactate plus citrulline malate (2 g/l); the respiratory intensities were measured in stationary regimen and in darkness, and were expressed as μmol of O_2 consumed/hr and per 10^6 cells.

ATP measurements: cell pools. ATP measurements were performed *in vitro*, by reference to a standard scale of ATP, in a "Nucleotimeter 107 CLV-Interbio" with lucioline (IB 20 IC CVL-Interbio) dissolved in a buffer, pH 7.75 (IB 25 50 CVL-Interbio) on 250 μl cell suspensions containing 25 μl of nucleoline (IB 40 50 CVL-Interbio) extemporaneously added. Nucleoline is a complex detergent which induces the total permeability of cellular membranes to nucleotides. Calculations of the ATP quantities were performed using the "peak condition" mode and 30 sec of preselection. The results were expressed in pmol of ATP per 10^6 cells, sampled at different phases of their growth in 33 mM lactate, in the absence or presence of citrulline malate.

On the three types of cells, after the first day of growth, the effect of an extemporaneous H_2O_2 (10^{-10} M) addition, on the cell ATP content was tested, and changes in ATP were expressed as percent of the cell ATP pool, taken as basis (Calvayrac *et al.*, 1985).

Ultraviolet irradiation effects on ATP pool

The three types of cell suspensions, after the first day of growth, were irradiated, for 1.5 min, with u.v.-light (Hg Vapor Zeiss lamp, high pressure HBO) at an incident energy of 5.10^{-2} Watt/cm^2, measured with a ISCO-spectroradiometer. The changes in ATP pools were then determined and the effect of an extemporaneous addition of H_2O_2 (10 M) was also tested. All ATP changes were then expressed as percentage of the cell ATP pool, taken as basis.

Effect of cyanide addition on ATP pools

In the three types of cell suspensions, after the first day of growth, 1 mM of KCN was added and ATP pools were determined before and after 1.5 min of u.v. irradiation. All ATP changes were then expressed as percentage of the cell ATP pool, taken as basis.

RESULTS

Preliminary experiments of cell growth in 33 mM lactate medium, suggested that the addition of 0.5 g/l and 2 g/l of citrulline malate induced different effects. We have consequently selected these two concentrations for our studies.

Cell growth

The study of the disappearance of lactate from the medium, Figure 1A, indicated that the citrulline malate at 0.5 g/l speeded the lactate assimilation. For instance, after 2 days of culture there was half the remaining lactate in the culture supplemented with 0.5 g/l of citrulline malate than in the control culture. On the contrary, at the concentration 2 g/l, the complex acted as a competitive substrate towards the lactate inducing delays in its metabolization as well as in cellular divisions (Fig. 1B).

The action of citrulline malate on *Euglena gracilis* growth is shown in Fig. 1B. The concentration 0.5 g/l did not modify the culture growth by reference to the control; on the contrary, the concentration of 2 g/l seemed to delay the cell divisions by provoking a slight latent phase and delaying the stationary phase of growth, which was still not attained after 7 days of culture. The control and the 0.5 g/l cultures, reached maximum after 3–4 days.

Measurements of cell proteins, Fig. 1C, and of intracellular paramylum, Fig. 1D, strengthened the hypothesis that 2 g/l of citrulline malate played the role of substrate. After 2 days of culture, the cells grown with 2 g/l of citrulline malate accumulated excess protein (70%) and paramylum (40%) and these excesses persisted almost unchanged between 2 and 3 days. The cells then presented comparable concentrations in both proteins and paramylum with control cells when they reached the stationary phase of growth. The comparison of Figs 1C and 1D indicated that if the concentration of 0.5 g/l of cit-

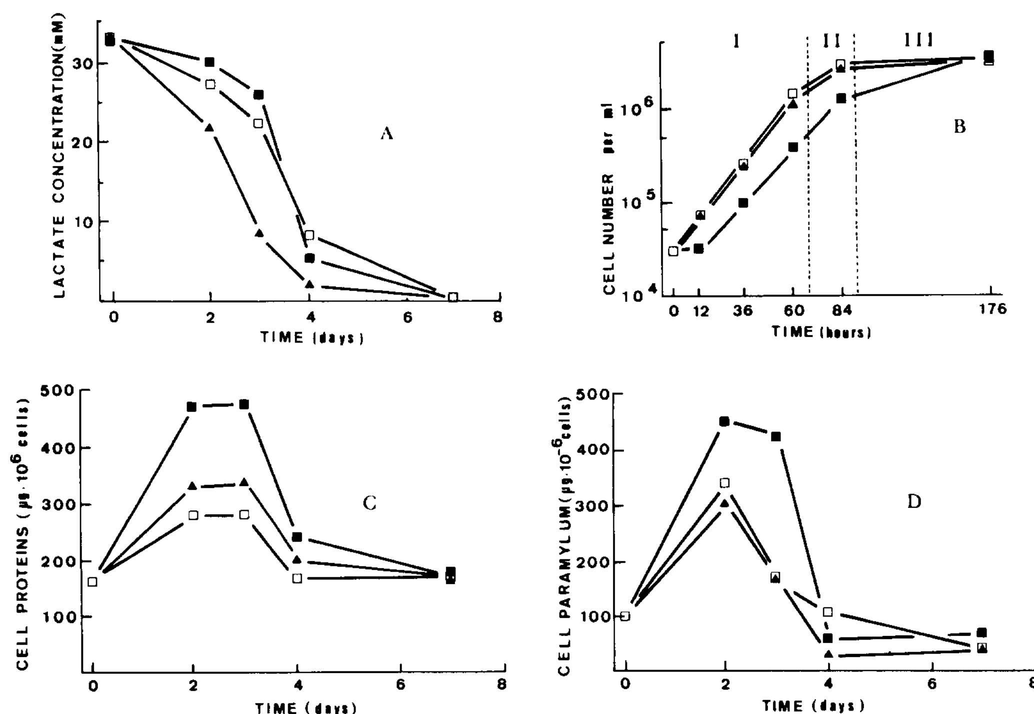

Fig. 1. *Euglena* cells were inoculated from the same preculture, and grown either in a lactate 33 mM medium (control, □), in lactate 33 mM plus 0.5 g/l (▲) of citrulline malate and in lactate 33 mM plus 2 g/l (■) of citrulline malate. (A) Disappearance, during the culture growth, of the lactate from the medium in the absence (control, □) or in the presence of 0.5 (▲) and 2 g/l (■) of citrulline malate. (B) Growth curves of three *Euglena gracilis* cultures, Phase I: exponential phase of growth; phase II: transition phase; phase III: stationary phase. (C) Variations, as a function of the age of the cultures, of the total proteins in cells growing in the absence (control, □) or in the presence of 0.5 (▲) and 2 g/l (■) of citrulline malate. (D) Variations, as a function of the age of the cultures, of the polysaccharide reserves (paramylum) stored inside cells growing in the absence (control, □) or in the presence of 0.5 (▲) and 2 g/l (■) of citrulline malate.

rulline malate did not affect the paramylum reserves, it provoked a slight transitory accumulation of cell proteins. In the presence of citrulline malate at 2 g/l, acting as competitive substrate, this process was retarded by 1 day. It was noteworthy (comparison between the Figs 1A and 1D) that the degradation of sugar reserves always began before the total exhaustion of the carbon substrate, the lactate.

Carboxylations and decarboxylations in the cells

It was important to study the carboxylation and decarboxylation phenomena since they played an important role in the polysaccharide synthesis both in hepatic cell (Di Tullio *et al.*, 1974) and in *Euglena* (Briand *et al.*, 1981).

After the first day of culture, the cells grown in the presence of citrulline malate, whatever the concentration tested (0.5 or 2 g/l), fixed the $^{14}CO_2$ more actively than the control cells, Fig. 2A. Nevertheless these intense carboxylations do not participate (Fig. 2B) in the important synthesis of paramylum (Fig. 1D). It was possible to observe a reinforcement of carboxylations after the third day, in the presence of 0.5 g/l and on the third day in presence of 2 g/l of citrulline malate (Fig. 2A), corresponding to the maximum in cell paramylum (Fig. 1D).

The labelling from the $^{14}CO_2$ used as tracer, detected in the glucide reserves, paramylum (β-1,3-glucan), Fig. 2B, suggested a strong participation of carboxylation for paramylum synthesis in the presence of citrulline malate (particularly at the dose of 0.5 g/l), during the first day of the culture. As soon as the second day this participation became lower in citrulline malate treated cells than it was in medium containing only lactate. After the third day there was no more noticeable effect of the citrulline malate and the participation of carboxylation in the paramylum synthesis diminished and evolved as in the control cells. Carboxylations were always accompanied by decarboxylations, indicating quick and active steps in anaplerotic, TCA cycle and synthesis pathways, leading from lactate to paramylum accumulation. Nevertheless whatever the added concentration of citrulline malate, decarboxylations seemed to be generally intensified, Fig. 2C, especially while the cultures reached the stationary phase (cf. Fig. 1B), at the difference of the control culture in which the decarboxylations remained relatively low. In stationary phase, the total quantities of ^{14}C fixed by the cell remained about constant since carboxylation as well as decarboxylation increased.

Cell respiration

The cell respiration was followed for each of the three types of cultures and for the duration of the culture growth. The evolution of the respiratory

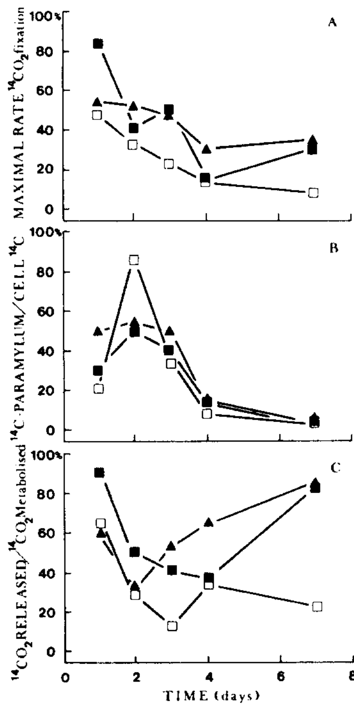

Fig. 2. Using ^{14}C-bicarbonate as tracer (see Materials and Methods), study of carboxylations and decarboxylations in cells grown in 33 mM lactate medium in the absence (control, □) or in the presence of 0.5 (▲) and 2 g/l (■) of citrulline malate. (*A*) Maximal rates of ^{14}C incorporation by the cells, as a function of culture age, the metabolized carbon (^{14}C found inside the cells plus $^{14}CO_2$ originating from decarboxylations) was expressed as per cent of the total radioactivity given by hour and by 10^6 cells (i.e. ^{14}C from the supernatant, from the cell pellet and from the Oxifluor); (*B*) ^{14}C stored in the polysaccharide reserves (the paramylum), as a function of culture age, expressed as percent of the cellular ^{14}C metabolized by the cells during the three hours of the incorporation experiments. (*C*) Variations of the cellular decarboxylations, as a function of culture age, expressed as per cent of the total ^{14}C metabolized during the 3 hr of the incorporation experiments.

intensities indicated (Fig. 3) that the cells grown in the presence of citrulline malate always exhibited higher respiration than the ones grown in the presence of the lactate alone. The intensification of respiration followed the dose of citrulline malate.

Cellular ATP

Determinations of cellular quantities of ATP indicated direct correlations between the presence in the medium of citrulline malate and the cellular energy. In the exponential phase of culture growth, after 1 day, the *in vitro* measurements of ATP pools were two and three times higher in the cells grown in the presence of 0.5 g/l and 2 g/l of citrulline malate than in the control cells (Fig. 4). Later on, after 3 or 4 days, the divergence damped down to stabilize to low values in all types of culture (about 10 pmol of ATP/10^6 cells).

At the beginning of the culture growth, when the cell numbers were about 0.1 10^6/ml, aliquots were sampled and cells transferred to the trizma buffer at a concentration of 10^6 cells/ml. The effects of H_2O_2

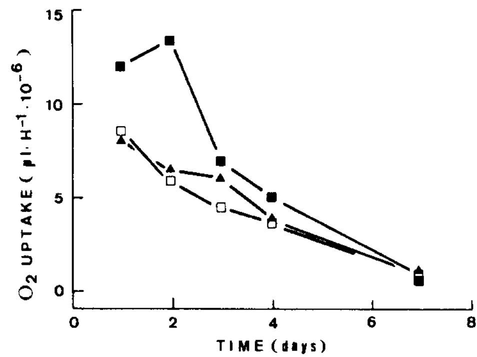

Fig. 3. Variations of the respiratory intensities, as a function of culture age of *Euglena* cells inoculated from the same preculture, and grown either in a lactate 33 mM medium (control, □), in lactate 33 mM plus 0.5 g/l (▲) of citrulline malate and in lactate 33 mM plus 2 g/l (■) of citrulline malate.

addition, of u.v. irradiation (for 1.5 min), and of KCN addition (1 mM; concentration three times lower than the one inhibiting respiration, and which according to Calvayrac *et al.*, 1985, is able to intensify the u.v. effect on ATP production) were tested on the cell ATP pools. The results obtained, reported in Table 1, clearly indicated that control cells, which presented a slight (28%) ATP increase in the presence of 1. 10^{-10} M H_2O_2, were insensitive to both u.v. irradiation and KCN addition. On the contrary, when cells were grown in media supplemented with citrulline malate, they exhibited important increases in ATP pools after 1.5 min of u.v. irradiation. These ATP increases were still present, and as intense, after KCN treatment. It was also striking to observe the direct correlations between the reinforcements of these phenomena and the citrulline malate dose.

DISCUSSION

Previous studies (Briand *et al.*, 1985) showed that the ultrastructural characteristics of the cells, particularly these of the mitochondria, were never affected by the presence of citrulline malate.

Citrulline malate, when added at the concentration of 2 g/l, contributed 40% of the exogenous carbon

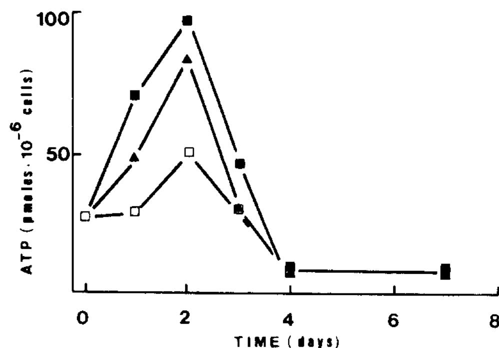

Fig. 4. Measurements, in cells grown in 33 mM lactate medium in the absence (control, □) or in the presence of 0.5 (▲) and 2 g/l (■) citrulline malate, of cellular quantities of ATP, as a function of the cell age. Results are expressed in pmol per million of cells.

and 72% of the exogenous NH_4^+, the 60% of remaining carbon being brought by the lactate, and the remaining 28% of NH_4^+ by the $(NH_4)_2HPO_4$ contained in the medium. In such conditions of culture the polysaccharide reserves of the cells increased in 48 hr, (Fig. 1D) without the intervention of carboxylation phenomena, which were still intense (Fig. 2A) but did not contribute noticeably to paramylum synthesis (Fig. 2B). Cells were enriched in proteins because of the NH_4^+ from citrulline, Fig. 1C; and the ATP quantities were remarkably reinforced on a per cell basis, (Fig. 4). The citrulline malate then acted as a competitive substrate towards the lactate, since there was a high cell content in paramylum (Fig. 1D), without simultaneous consumption of the lactate from the medium (Fig. 1A). Nevertheless a slight latent phase and a delay of cell divisions could be observed, which led to a later stationary phase. Intense decarboxylation accompanied such a metabolism, occurring probably from the rapid turnover of intermediary molecules, but not from reserves as paramylum, very weakly labelled (Fig. 2B). Such results indicated that, according to the scheme proposed in Fig. 5, citrulline malate was immediately metabolised: the citrulline contributing to protein synthesis via the urea cycle; and the malate being quickly used, either by directly entering the tricarboxylic acid cycle, or by giving paramylum reserves without intervention of the anaplerotic pathway of carboxylation (which normally synthesizes OAA by carboxylation of the PEP formed from the lactate via the pyruvate, see Fig. 5). The anaplerotic pathway for paramylum synthesis appeared only on the third day, Fig. 2A and B, when lactate exhaustion was speeded up. We propose the interpretation presented in Fig. 5 and according to which, in the presence of an excess of citrulline malate, 2 g/l, the TCA cycle was probably slowed down and malate gave an excess of OAA directly leading to paramylum accumulation; consequently the amount of OAA formed by carboxylation of the PEP was reduced.

At lower concentration, 0.5 g/l, when citrulline malate participated only for 15% of the exogenous carbon and for 40% of the NH_4^+, it acted synergistically on the lactate assimilation, nevertheless

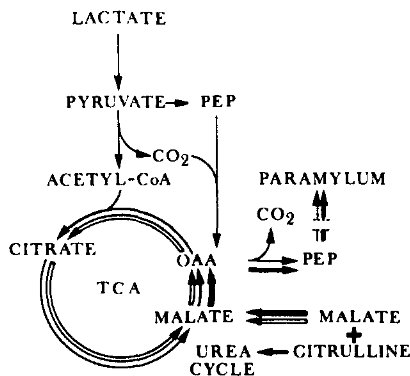

Fig. 5. Scheme of the metabolization of lactate by *Euglena gracilis*. Arrows indicate the possible modifications produced by citrulline malate, according to our results, on such a metabolism. Black arrows: 2 g/l of citrulline malate; hollow arrows: 0.5 g/l of citrulline malate.

Table 1. Cell ATP pools, i.e ATP basis in pmol per 10^6 cells, in *Euglena gracilis* Z, grown for one day on media: 33 mM lactate (control); 33 mM lactate plus 0.5 g/l citrulline malate (0.5 g/l C.M.); 33 mM lactate plus 2 g/l citrulline malate (2 g/l C.M.). Effects, on the ATP cell amounts, of either the addition of 10^{-10} M of H_2O_2, or a u.v. irradiation of 1.5 min, or a KCN (1 mM) treatment. The changes were expressed as percent of the ATP basis

Culture conditions	Control	0.5 g/l C.M.	2 g/l C.M.
ATP basis (pmol per 10^6 cells)	28	48	72
H_2O_2 10^{-10} M	+28%	−15%	+6%
u.v. irradiation (1.5 min)	−4%	+54%	+117%
u.v. irradiation + H_2O_2 10^{-10} M	−20%	+39%	+128%
KCN (1 mM)	+7%	+16%	+42%
KCN (1 mM) + u.v. irradiation	0%	+29%	+109%

without affecting the curve of cell growth (Fig. 1B). The disappearance of the lactate from the medium was drastically accelerated (Fig. 1A), without simultaneous accumulation of labelled paramylum (Fig. 2B). The carboxylation might be furnishing intermediary molecules which, after 3 days, either led to paramylum synthesis (Fig. 2B), or were decarboxylated (Fig. 2C). The action of such a low citrulline malate concentration could be interpreted, according to Fig. 5. The malate might enter and accelerate the TCA cycle; this could explain the remarkable ATP pool then measured (Fig. 4) in these cells enriched in proteins (Fig. 1C). In such conditions of culture citrulline malate seemed not abundant enough to play the role of a competitive substrate towards the lactate but on the contrary, acted synergistically.

Concerning cellular energetics and taking into account the carbon equivalents in each of the three used culture media, the addition of 0.5 and 2 g/l of citrulline malate brought respectively 18 and 66% of excess carbon by reference to the control medium containing only lactate. It was interesting to notice, (Fig. 4), that if the ATP quantities per cell were doubled in the presence of 0.5 g/l of citrulline malate, during the first 2 days of the culture; they only slightly differed between the cells grown with the concentrations 0.5 and 2 g/l of citrulline malate. Nevertheless, it seemed that this remarkable amount of ATP found in cells grown in medium containing citrulline malate, which corresponded to relatively high respiratory intensities (Fig. 3), could still not be explained by a direct correlation between the O_2 consumed and the ATP formed. We suggest that the reinforcement of ATP pools, in the citrulline malate grown cells, could be due to the additional functioning of the secondary respiratory pathway, insensitive to cyanide, the existence of which had been demonstrated in Table 1.

In *Euglena*, it had been previously demonstrated: on the one hand, the existence of an alternate respiratory pathway, which did not use the oxidase cytochrome and was consequently insensitive to cyanide (Calvayrac *et al.*, 1971, 1978); and on the other hand of a photoconsumption of oxygen stimulated by u.v. light (Calvayrac and Laval-Martin, 1980) able to produce ATP by dismutation of H_2O_2 by catalatic-type activities (Calvayrac *et al.*, 1985). It seemed interesting to consider our results according to the

following hypothesis that citrulline malate added to the culture medium could reinforce such a special pathway and consequently produce the excess of cellular ATP. The study of the ATP pool evolutions under u.v. light (Calvayrac *et al.*, 1985), and tested here in the absence or presence of KCN 1 mM, indicated (Table 1) a reinforcement of both the insensitive to cyanide pathway and the u.v. promotion of ATP synthesis, in cultures grown in the presence of citrulline malate per reference to control conditions. It appeared reasonable to ascribe to a reinforcement of the catalatic type activities, the excesses of ATP measured in cells grown in the presence of citrulline malate (especially at 0.5 g/l, Fig. 4).

These studies on the cellular and subcellular effects of citrulline malate did not demonstrate any toxic effect of this complex on *Euglena* grown in the presence of 33 mM lactate as carbon substrate. On the contrary they seemed to illustrate a synergistic effect of this product, at the concentration of 0.5 g/l, on the lactate assimilation. It was then possible that the rapid recovery, after an intense muscular effort, of people treated with citrulline malate who presented a fast disappearance of blood lactate (Fornaris *et al.*, 1984), could be due to the quick degradation of blood lactate by the hepatic cells, accompanied or not by glycogen accumulation. Perfusions of rat livers with lactate or lactate plus citrulline malate solutions might be performed in order to test such an hypothesis. Intraperitoneal perfusions done on mice have recently shown (Astoin, personal communication) that citrulline malate at the dose of 0.2 g per 100 g of fresh wt, provoked a significant augmentation of the life-time when the subjects were treated by cyanide; put in hypoxy cytotoxicity; subjected to altitude or deprived in oxygen.

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