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USE OF A MICROBIAL MODEL FOR THE DETERMINATION OF DRUG EFFECTS ON CELL METABOLISM AND ENERGETICS: STUDY OF CITRULLINE-MALATE

JOËL BRIAND,† HENRI BLEHAUT,* RÉGIS CALVAYRAC AND DANIELLE LAVAL-MARTIN

Laboratoire des Membranes Biologiques, Université Paris 7, Tour 54–53, 2 Place Jussieu, F-75005 Paris
and
**Centre de Recherche des Laboratoires Biocodex, 19 rue Barbés, 92120—Montrouge, France*

ABSTRACT

Euglena gracilis can be used as a microbial model to study the effect of drugs on lactate metabolism and gluconeogenetic synthesis. The cell growth and metabolism have been characterized in a 33 mM lactate medium, non-supplemented or supplemented by *dl*-malate or by *l*-citrulline alone or by the compound formed by the stoichiometric combination of the two components: the citrulline-malate (Stimol®). The malate of the complex accelerated the ammonium disappearance, while the citrulline facilitated the lactate consumption. A synergistic action of the complex, by comparison with the additive effects of the individual components, on most of the parameters studied was detected. A remarkable resistance to anoxia, and a quicker recovery under aeration of the cells supplemented with CM, were evident: after carbonation for 2 min the total nucleotides in the medium were increased by 44 per cent with an unchanged energy charge; and after a prolonged (20 min) anoxia followed by an aeration, the capacities of the cells to synthesize ATP in the presence of excesses of both ADP and phosphate were two-fold higher in Stimol® treated cells than in control.

KEY WORDS Citrulline-malate Anoxia Cell energetic Microbial model.

INTRODUCTION

The need to find alternatives to animals in medical experiments has led to the utilization of microbial cell cultures to gain understanding of the effects of chemicals on cells isolated from the complex interactions of the intact multicellular organism.[1–6] Because experiments on animals are very expensive, cell cultures have become invaluable for screening the effects of drugs; they can generate significant data because the culture conditions are well defined, and

† Addressee for correspondence.

ABBREVIATIONS: C: citrulline; CM: citrulline-malate, stoichiometric complex (Stimol®, Biocodex, France); cyt P-450: cytochrome P-450; L: lactate; M: malate; OAA: oxaloacetic acid; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PEP: phosphoenol pyruvate.

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the abundant cell populations are homogeneous. *Euglena gracilis* is an interesting microbial model of mammalian hepatic metabolism since the processes which transform lactate in polysaccharide reserves in this unicellular protist[7] and in hepatic cells[8,9] are almost identical. The mitochondrial OAA, originating from the carboxylation of either PEP in *Euglena gracilis* or pyruvate in liver cell, is decarboxylated in both types of cells by a membranal and specific enzyme. This enzyme, the PEP carboxykinase guanosine triphosphate (GTP) dependent (E.C.4.1.1.149), translocates the resulting PEP out of the mitochondria for the subsequent gluconeogenesis. In the hepatic cell, glycogen is formed on a cytoplasmic promotor;[9] in *Euglena gracilis*, the β-1,3-glucan (paramylum) is stored in mitochondrial vesicles.[10] Moreover, similar effects of ethanol on the compositions of microsomal fractions extracted either from *Euglena* or from rat liver have been demonstrated,[11] i.e. decreased quantities of fatty acids, inversely proportional changes in the PE and PC, and augmentation of the total of cyt P-450. Very recently, immuno-blotting analysis (Western blots) has demonstrated the presence of several isoenzymes of cyt P-450 in *Euglena*. These were recognized by antibodies of either human or rat cyt P-450 (article in preparation).

The citrulline-malate (Stimol®, Biocodex, France), CM, complex obtained by the stoichiometric combination of the two components is usually administered to athletes for quick recovery from fatigue[12] or as a treatment of physical asthenia in convalescent people.[13–17] Both symptoms have been characterized by increases in the blood concentrations of lactate[18] during the ATP reformation by anaerobic glycolysis[19] and of ammonium,[20] as well as by a breakdown of high energy phosphate compounds.[18] Conversely, recovery from physical effort has been correlated to lactate disappearance from blood.[21] A study, at the cellular level, of the possible modifications induced by CM on lactate metabolism and gluconeogenetic synthesis on the one hand and of the cell energetics on the other hand, seemed necessary. Using *Euglena* as a microbial model, we have already shown that the addition of 1·5 mM of CM (equivalent to the typical therapeutic concentration in the blood) to the 33 mM lactate (L) culture medium, induced an increased rate of disappearance of the lactate from the medium, accompanied by increases in both cell carboxylations and ATP pools.[22] The highest dose of CM (6 mM) acted as a competitive substrate.[22]

The aim of this study was to establish the roles of each of the components of CM, when added individually to the L medium (in exactly the same quantities contained in the stoichiometric compound when used at 1·5 mM). Several parameters were monitored as a function of the age of the culture, i.e. (1) cellular growth; (2) rates of consumption, per hour and per million cells, of the main components of the culture medium (lactate, ammonium, and when present, malate and citrulline); (3) rates of synthesis and subsequent degradation, per hour and per million cells, of the main cell components (proteins, lipids, paramylum); (4) urea excretion; (5) respiratory intensity; (6) nucleotide pools and energetic characteristics, as well as the changes occurring in these parameters, after

a 2 min aeration or a 2 min anoxia (by carbonation) of the cells. The resistance of cells grown with CM to anoxia was further investigated.

MATERIALS AND METHODS

Euglena gracilis wild strain Z (n°1224–5d, Cambridge, England) was grown, without agitation, in darkness at 27°, in a 2 l toxin flask containing 1 l of liquid mineral medium,[7] 3·7 mM ammonium, and 33 mM lactate (L medium) supplemented with B_1 and B_{12} vitamins; the pH was 3·5. Media were autoclaved at 110° for 30 min. When added at final concentration of 1·5 mM: the *dl*-malate (L + M medium); the *l*-citrulline (L + C medium) or the citrulline-malate complex (L + CM medium), were injected through sterile Millipore filters prior to the cell inoculation. Initial cell titres were standardized to 3×10^4 per ml; L cultures which were 3 days old were used as the preculture for inoculation. The four types of cultures (L, L + C, L + M, and L + CM) were examined every 12 h for the first 4 days after inoculation, and then on the seventh day. In order to gather 2×10^7 cells, the volumes sampled varied from 50 to 200 ml. This abundant and homogeneous cell population allowed quantifiable data to be obtained at each stage of culture growth.

Cell growth

The cell titre was determined, on a Malassez cell (0·2 ml on 5 mm^2), after dilution and immobilization of the cells by potassium iodide (5 to 8 per cent final).

Biochemical measurements of the medium components

Lactate (Serva) and *dl*-malate (Merck) concentrations, respectively, were determined by following spectrophotometrically the $NADH + H^+$ produced when lactate dehydrogenase transformed lactate to pyruvate[23] and when malate dehydrogenase transformed *l*-malate in OAA.[24] Using HPLC analysis, initial tests had shown that:

1. the *d*- and *l*-lactate forms were in the respective proportions of 3:1 after autoclaving;
2. during the growth of the culture, these two racemic forms were consumed at the same rate;
3. the two racemic forms of *dl*-malate produced a unique peak at time 0, which decreased with cell growth.

Citrulline concentrations were determined spectrophotometrically at 530 nm using a colorimetric method. After condensation of 0·2 ml of the citrulline solution with diacetylmonoxime (1 ml of 0·05 per cent diacetylmonoxime, and

0·001 per cent thiosemicarbazide acting as accelerator) in an acidic oxidizing medium (2 ml of 8·5 per cent H_3PO_4, 30 per cent of concentrated H_2SO_4, and 0·01 per cent of $FeCl_3$, $6H_20$ in a boiling water bath for 5 min), the reaction mixture was equilibrated for 10 min in darkness at room temperature prior to measurement. This method gave linear calibrations from 30 to 120 μM. Ammonium ion concentration was measured by following the $NADH + H^+$ consumption occurring when glutamate dehydrogenase transformed the 2-oxoglutarate in *l*-glutamate.[25] The concentration of urea was determined from the measurements of the ammonium generated after urease treatment (10 min at 25°), a correction being made for the initial ammonium ion content.

Extraction and measurements of cell components

Cell suspensions were centrifuged for 10 min at 1500 g (Sorvall RC2B). The 20×10^6 pelleted cells were resuspended in 1 ml water and then sonicated twice (Measuring Scientific Equipment Ltd, England) at 4° for 15 s (8 kHz). Aliquots from these sonicates were used for either protein, lipid or paramylum determinations.

(a) Protein content from 1×10^6 cells was measured according to Bradford,[26] the standard curve being established with bovine serum albumin.
(b) Lipid content from 1×10^6 cells was determined chemically with vanillic acid.[27]
(c) Paramylum was extracted by 30 per cent KOH from 1×10^7 cells, precipitated by absolute ethanol, and then measured using anthrone.[28]

Statistical significance of the results

In order to support the use of this microbial system as a metabolic model, a study of its stability was necessary. Cell titres and biochemical parameters were determined throughout the growth of individual control (L) cultures by either successive samplings from the same five flasks or single sampling, in turn from a set (50) of flasks, at each time point. The standard deviations did not exceed ±5 per cent for each of the parameters analysed.

The experiments using citrulline-malate components were repeated three times; they indicated standard deviations of *c.* ±4·5 per cent comparable to those of the controls, i.e. ±4·9 per cent; the results were pooled for the computation of the mean curves.

Biochemical data

The raw data on cell densities, the concentrations of each component found in the medium, and the intracellular concentrations of proteins, lipids or paramylum, are presented in Table 1. These data were then fitted, the standard deviations being taken into account, with a non-linear regression method

(GraFit, Erathicus Software Limited, 1989; Graphpad, ISI Software, 1987), generating the most probable intermediary values corresponding to each hourly interval. Then, for every successive 1 h interval (t_2–t_1), the component concentration (C_2–C_1) consumed (or produced) by the number n_2 of cells per hour was expressed per million cells present at time t_2; the rate curves were then computer generated. They represented the variations of either the rates of substrate consumption or excretion, or of the rates of cellular synthesis or degradation, per hour and per million cells as a function of the culture age. Such rates allowed us to follow the dynamics of the cellular metabolism, when substrate concentrations and cell populations varied simultaneously, and to distinguish the sequence of metabolic events.

Respiratory activities

The respiratory activity was measured polarographically with a Clark type electrode, at 27°, in stationary regimen and in darkness. They were expressed as μmol O_2 consumed per hour and per 10^6 cells.

Nucleotides measurements

ATP, ADP, and AMP concentrations, in cells from L, L + M, L + C, and L + CM, sampled when the cell titres reached $1{\cdot}0$–$1{\cdot}5 \times 10^5$ per ml of culture medium, were measured before and after, either aeration by compressed air, or carbonation for 2 min (flow rates of 10 ml CO_2 per min). One milliliter of cell suspension was fixed by 0·1 ml perchloric acid at 4°; the mixture was kept at 4° for 20 min and shaken often; it was then centrifuged in an Ependorf centrifuge tube (maximal speed for 1 min). The supernatant was neutralized by 1 ml trioethylamine and then mixed using a vortex generator for 1 min with 1 ml freon 112. Centrifugation at 3000 g for 5 min allowed three phases to be decanted: the aqueous epiphase (500 μl) containing all the nucleotides was sampled and frozen at −20° until just prior to the determination of nucleotide levels by *in vitro* measurements of the fluorescence of an acellular preparation from firefly lanterns. The results were expressed as nmol of nucleotide per 10^6 cells. The standard errors calculated on the basis of triplicate experiments were ±10 per cent. [29]

Another type of measurement indicated the capacity of the cells to synthesize ATP, in the 30 s period immediately following treatment by a detergent, which rendered the cells permeable to nucleotides and the added ADP and PO_4^{-3}. This method used a Nucleotimeter 107 (CLV-Interbio), set on 30 s of preselection and 'peak condition mode', using 100 μl of luciferin-luciferase mixture from firefly lanterns (4L Biosys reactive) dissolved in water and injected into a mixture of 100 μl of cell suspension in Trizma base buffer 50 mM, pH 7·2, containing 2 mM $MgCl_2$ and 2 Mm $CaCl_2$ (cell titre standardized at 10×10^6 cells per σml), 20 μl of ADP 5×10^{-6} M, 10 μl of PO_4^{-3} 5×10^{-4}M, 30 μl water and

Table 1. Changes in mean ($n = 3$) cell densities, concentrations of added substrates and intracellular concentrations of proteins, lipids, and paramylum with time, for cell grown in lactate 33 mM medium (control, L), or plus 1·5 mM malate (L + M), or plus 1·5 mM citrulline (L + C), or plus 1·5 mM citrulline malate (L + CM)

	Time (h)									
	0	12	24	36	48	60	72	84	96	168
Cell number (million ml^{-1})										
L	0·03	0·03	0·04	0·05	0·11	0·31	0·96	1·95	2·78	3·04
L + CM	0·03	0·03	0·04	0·04	0·12	0·34	0·92	2·05	2·70	3·00
L + M	0·03	0·03	0·05	0·07	0·13	0·38	0·85	1·85	2·55	3·20
L + C	0·03	0·03	0·03	0·04	0·07	0·24	1·08	2·55	3·16	3·20
Lactate ($g\ l^{-1}$)										
L	2·9733	2·8832	2·7931	2·6129	2·2525	1·3515	0·04505	0·03604	0·04505	0·04505
L + CM	2·9733	2·8832	2·6129	2·2525	2·0723	0·9911	0·04505	0·04505	0·03604	0·06307
L + M	2·9733	2·9733	2·7931	2·703	2·5228	1·3515	0·17119	0·05406	0·03604	0·08109
L + C	2·9733	2·9733	2·8832	2·703	2·5228	1·2614	0·02703	0·04505	0·07208	0·10812
Malate ($g\ l^{-1}$)										
L	0	0	0	0	0	0	0	0	0	0
L + CM	0·22	0·213	0·185	0·147	0·113	0·097	0·078	0·073	0·078	0·065
L + M	0·22	0·219	0·212	0·15	0·126	0·094	0·085	0·082	0·071	0·058
L + C	0	0	0	0	0	0	0	0	0	0
Citrulline ($g\ l^{-1}$)										
L	0	0	0	0	0	0	0	0	0	0
L + CM	0·28	0·258	0·23	0·204	0·196	0·188	0·201	0·153	0·122	0·177
L + M	0	0	0	0	0	0	0	0	0	0
L + C	0·28	0·272	0·264	0·24	0·223	0·211	0·208	0·151	0·131	0·127

Table 1 continues opposite

Table 1 continued

	Time (h)									
	0	12	24	36	48	60	72	84	96	168
Ammonium (g l^{-1})										
L	0·125	0·12	0·113	0·094	0·082	0·041	0	0	0	0
L + CM	0·125	0·111	0·099	0·082	0·073	0·036	0·001	0·002	0	0·1
L + M	0·125	0·108	0·093	0·084	0·073	0·033	0·002	0·002	0	0·003
L + C	0·125	0·125	0·125	0·112	0·089	0·043	0·001	0	0·001	0·008
Urea (g l^{-1})										
L	0	0·011	0·032	0·021	0·014	0·012	0·014	0·013	0·013	0·011
L + CM	0	0·007	0·028	0·032	0·042	0·026	0·014	0·014	0·013	0·006
L + M	0	0·015	0·038	0·047	0·055	0·028	0·012	0·015	0·014	0·007
L + C	0	0·014	0·038	0·031	0·024	0·016	0·012	0·012	0·013	0·007
Proteins (mg million^{-1})										
L	186	205	227	219	200	191	172	125	117	94
L + CM	186	218	247	221	195	171	160	138	116	96
L + M	186	198	214	220	217	156	128	125	119	109
L + C	186	231	253	235	211	149	117	118	115	112
Liquids (μg million^{-1})										
L	143	191	241	205	177	168	154	99	56	55
L + CM	143	182	240	209	187	152	143	81	54	54
L + M	143	215	272	289	332	241	115	92	77	82
L + C	143	287	431	352	229	145	96	71	65	52
Paramylum (μg million^{-1})										
L	198	359	496	421	352	263	204	142	112	60
L + CM	198	392	556	368	216	211	204	108	84	36
L + M	198	341	468	339	252	218	204	149	104	54
L + C	198	309	380	394	400	246	112	106	84	36

25 µl of extractor (complex detergent, Bi 5002). The ATP quantities, expressed in pmol of ATP per 10^6 cells, were calculated by reference to standards of ATP tested in an identical cocktail. The luminescence produced is proportional to the ATP concentration according to the reaction:

$$ATP + luciferin + O_2 \xrightarrow{\text{luciferase}} oxyluciferin + AMP + PPi + CO_2 + light$$

Such a method gave values about 50 times lower than the intracellular ATP concentrations.

Cells from L, L + M, L + C, and L + CM were sampled when the cell titres reached 1·0–1·5 10^5; they were concentrated to 1×10^7 per ml in the culture medium and then incubated for 2 h at 27° in the presence of either lactate alone (L), malate alone (M), citrulline alone (C) or citrulline malate (CM). The ATP contents of each type of cell were determined at time zero and after 2 min of aeration by compressed air or carbonation by bubbling of CO_2 (flow rates of 10 ml min^{-1}). In all cases, the cells had to be transferred in the Trizma buffer (at the same cell concentration) just prior to ATP determinations.

RESULTS AND DISCUSSION

Cell growth and changes in the mediums

The mean growth curves of the four types of cultures: lactate alone (L), lactate plus malate (L + M), lactate plus citrulline (L + C), and lactate plus citrulline-malate (L + CM), based on the data in Table 1, are presented in Figure 1(a). They are sigmoidal and indicate four distinct phases: a latence period of adaptation to the renewed substrate; an exponential phase of growth over the next 2–3 days, when cell titres were less than $1{\cdot}5 \times 10^6$ cells ml^{-1}; a transition phase of 1 day duration, when the substrate became depleted, with cell titres between 1·5 and $2{\cdot}8 \times 10^6$ cells ml^{-1}; and finally a plateau phase, corresponding to the maximal cell titre.

The growth curves were strictly identical for L and L + CM (generation times, $g = 10$ h); the addition of malate to the L + M medium, decreased the latence period from 30 to 18 h and slowed down the growth (generation time, $g = 16$ h). On the other hand, the addition of citrulline provoked a substantial increase in the latence period (36 h) and rate of growth ($g = 5$ h). There were similarities between the four types of culture; in each case the stationary, i.e. plateau, phase began on day 4 and the final stable cell titres were *c.* 3×10^6 cells ml^{-1}.

The rates of lactate consumption per hour and per million cells, as a function of the age of the culture, are shown in Figure 1(b); the actual data are given in Table 1. After 45 h of culture, the maximal rate was of 7·5 µmol h^{-1} per million cells in the control L culture; this rate was increased by 16 per cent in L + CM. This effect was apparently due to the citrulline and not to the malate moiety of the complex, since the addition of citrulline alone sped up

the rate of lactate consumption by 80 per cent (factor 1·8) compared with lactate alone. On the other hand, malate alone retarded the onset of the consumption by 20 h and decreased the maximal rate by 20 per cent and delayed the onset by 10 h. The time taken to reach half peak-height indicated that, except when malate alone was added, the lactate was largely metabolized in the 24–36 h period following inoculation. This period of intense metabolism corresponded to the exponential phase in the growth of the culture.

The rate of ammonium consumption, Figure 1(c) from the data of Table 1, was characterized in L, by a maximal value of 1·5 μmol h^{-1} and per million cells. The consumption was retarded by 24 h and the peak occurred after essentially the same time (48 h) in both L and L + C cultures, the rate characterizing the latter being more than double that for the L culture. The presence of malate either alone (L + M) or in the complex (L + CM) induced an earlier consumption of ammonium (peaks at about 35 h), the rate being 40 per cent greater with the complex. In all cases, ammonium is totally consumed within 72 h (see also data in Table 1).

The peak rates of consumption of the malate and/or citrulline, initially present at the concentration of 1·5 mM (Figure 1(d) from the data of Table 1), both occurred after about 30 h in the L + CM system. In the L + M culture, the maximum rate of malate consumption occurred at 38 h. In the L + C culture, the maximum rate of citrulline consumption occurred after 40 h. The maximal rate of malate consumption was 50 per cent greater in L + CM than in L + M. On the other hand, in L + CM the rate of consumption of citrulline was slightly depressed. The shapes of the curves, as well as the times taken to reach half peak-heights, indicated that the malate was consumed more rapidly than the citrulline. These two components were never totally consumed, the final concentrations remaining at 0·43 mM malate and 0·65 mM for citrulline.

Formation of the main cellular components

The effects of the CM complex on cell metabolism, as a function of the age of the culture, were determined by using the data in Table 1 and calculating the rates of synthesis or degradation of the proteins (Figure 2(a)), lipids (Figure 2 (b)), and paramylum (Figure 2(c)).

By comparison with L, the rate of protein synthesis (peaking at 13 h) was increased by a factor of 2 in L + C and 2·5 in L + CM (Figure 3(a)). This effect was obviously due to the citrulline, since the maximal rate of synthesis, although similar in value, occurred 5 h later in L + M than in L. After about 30 h, the turnover of proteins was more or less in equilibrium in the four types of cultures. Later, protein degradation occurred; this began almost simultaneously in cells cultured in L, L + CM, and L + M but occurred earlier in L + C. The rates of degradation were of the same order in cells from L and L + CM, but much more rapid in the presence of malate and citrulline alone. The durations of the degradation phases, estimated from the time to half peak-

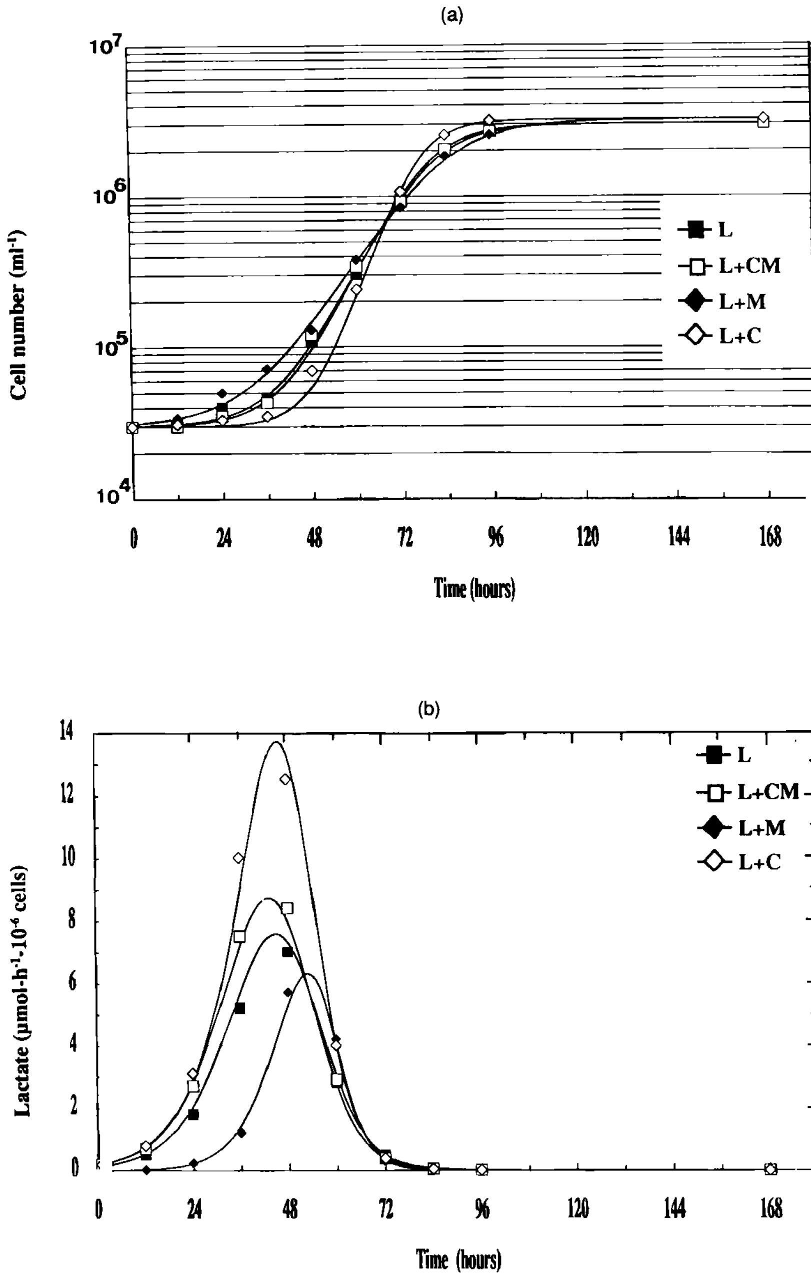
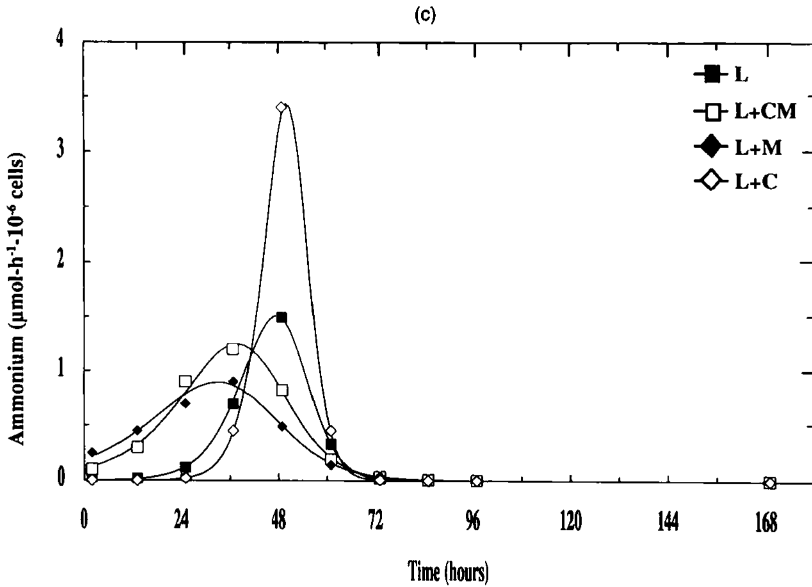
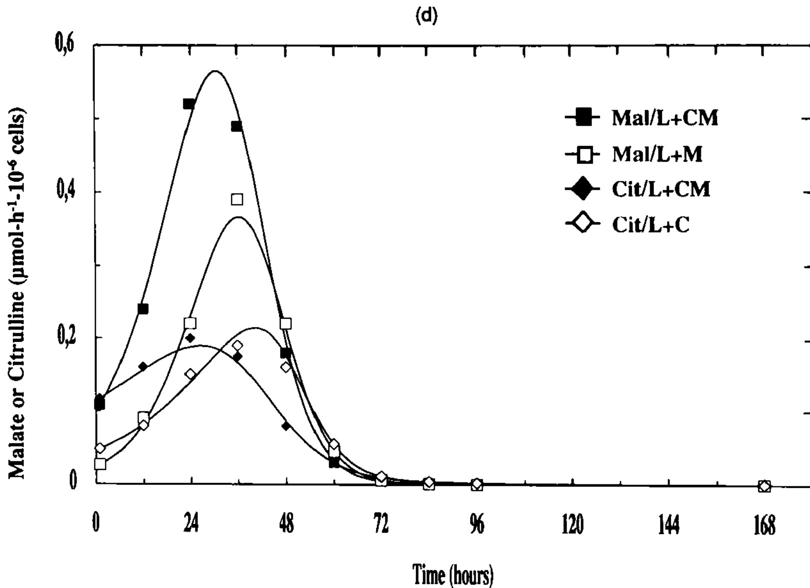


Figure 1. Best fit curves generated from the data in Table 1, for (a) cell growth, (b) utilization of lactate, (c) utilization of ammonium, (d) utilization of malate and citrulline, in the control (L), L + M, L + C and L + CM systems, as a function of time.





height, in the L + M and L + C cells (15–20 h) were about half those of the cells grown on L or L + CM (40 h). Quantitatively, the protein contents varied from 180 μg per 10^6 cells at time 0 to 100 μg at time 120–168 h, when cell division stopped completely (Table 1).

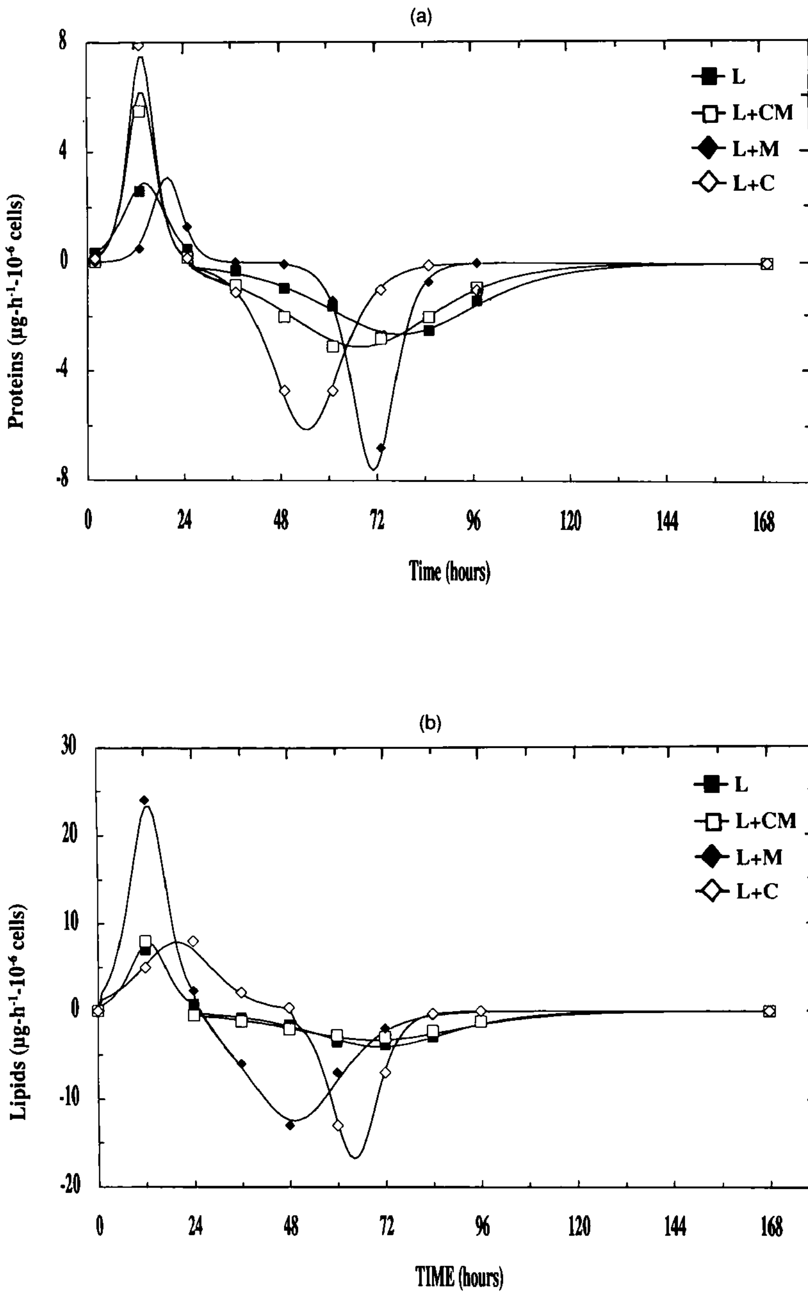
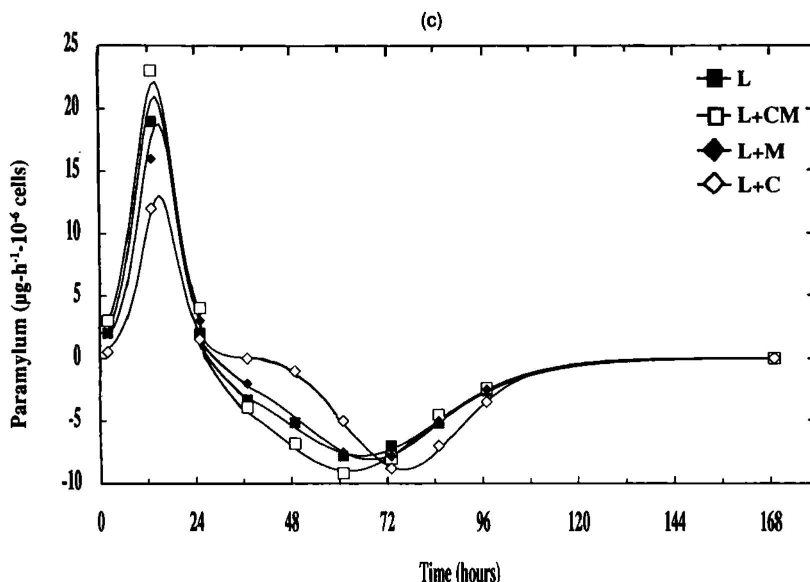


Figure 2. Best fit curves, based on the experimental data in Table 1, for the generation and utilization of (a) cell proteins, (b) cell lipids, (c) cell paramylum, in the control (L), L + M, L + C, and L + CM systems, as a function of time



The complex had no significant effect on the generation of lipids (Figure 2(b)) since the L and L + CM curves were essentially superimposable, lipid synthesis peaking at 13 h (Figure 2 (a)), followed by a slow (40 h to half peak-height) degradation. The malate, as evidenced by the L + M system, provoked intense lipid synthesis, peaking at 13 h, as with the L cells, but three-fold more intense; a relatively slow degradation followed. In the case of L + C, the maximal rate of synthesis corresponded with that for L cells, but took twice as long to achieve; subsequent degradation was twice as fast. Quantitatively, the lipid contents varied from 150 µg per 10^6 cells at time 0 to 65 µg at time 120–168 h, in cells which had ceased dividing (Table 1).

The synthesis of the cellular energetic reserves (the paramylum) from the lactate substrate in L, L + M, and L + CM was completed by the end of the first day (Figure 2(c)). It was followed, for 4 days, by a complete and almost identical course of degradation, characterized by a rate three times less than the rate of synthesis. Synthesis of paramylum by cells cultured in L + C medium occurred simultaneously, but with a rate reduced by 40 per cent which leads to a maximal accumulation which is 20 to 33 per cent less than obtained with the other three conditions. In cells from L + C, the degradation of paramylum was evidently more rapid than in the other cell types since, although onset was delayed by 15 h in L + C, degradation was complete after about the same time in each system.

Excretion and absorption of urea

In addition to substrate consumption (Figure 1(b), (c), and (d)) and to accumulation of cellular components, followed by degradation (Figure 2(a), (b), and (c)), urea was detected in the media. During the first day of growth, in all four types of media, urea (Figure 3, from the data of Table 1) was excreted in amounts reaching about 0·034 gl^{-1}; this was followed by an absorption of 70 per cent of the maximal quantities excreted. In L and L + M media (maximal rates peaking at time 10 h) the excretion began earlier than in the L + C and L + CM media (peaks at 18 h). The urea, excreted during the first day of culture growth in L and L + M media, was actively absorbed before the end of the second day from L medium; it was consumed more slowly from L + M. In L + C, the cellular absorption of the excreted urea stopped at the end of the third day of culture. In L + CM, unlike the other three media, absorption did not seem to occur, or only very slightly, suggesting that the rate of excretion was always equal to or greater than that for absorption, since it was, in fact, the net effect of the two processes that was measured.

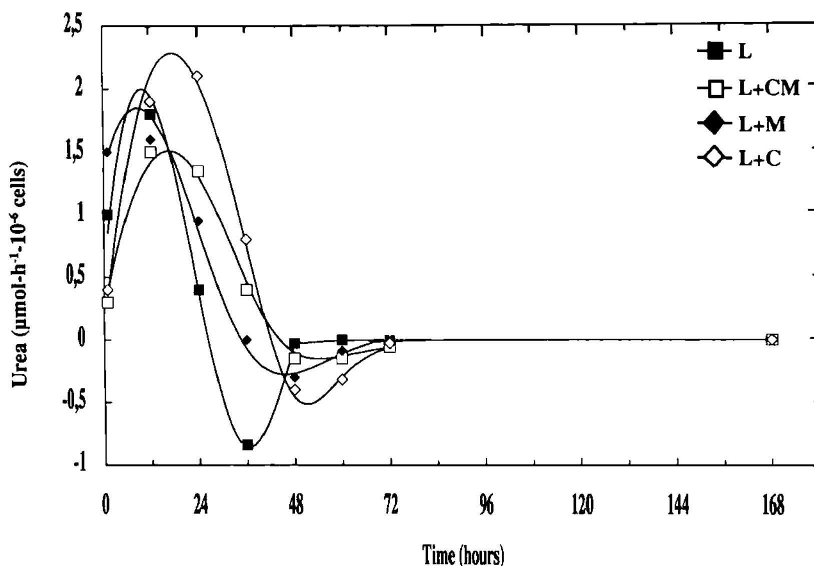


Figure 3. Best fit curves, calculated from the data in Table 1, for urea utilization in the control (L), L + M, L + C, and L + CM systems, as a function of time

Cell respiration

Relative to control cells grown in L, the respiratory activities during the first day of culture were decreased by 38 per cent in L + CM but only by

8 per cent in L + M (Figure 4). On the other hand they were reduced by 17 per cent in L + C. After day 2, the respiratory activities were identical in all four types of medium; they then decreased dramatically to reach very low values at day 4, by which time the remaining malate and/or citrulline levels in the media were steady (Figure 1(d)).

Nucleotide pools and energy charges

The measurements were performed on cells sampled at the beginning of the exponential phase of growth, after 2 days of culture; the cell titres were then about 1·0–1·5 × 10^5 per ml, and the respiratory activities were very high (Figure 4).

Table 2. Nucleotide and total nucleotide, Σ, contents (nmol 10^{-6} cells) and energy charge (E.C.) of *Euglena* cells, inoculated from the same preculture, and grown in the control medium (L) and in the malate (L + M), citrulline (L + C), and in citrulline-malate (L + CM) supplemented systems, determined when the cell titres reached 1·0–1·5 × 10^{-7} cells ml^{-1}

Cell system*	ATP	ADP	AMP	Σ	E.C.
L	1·13	1·21	0·16	2·50	0·69
L + M	0·69	0·66	0·08	1·43	0·71
L + C	1·11	1·00	0·18	2·29	0·70
L + CM	1·25	0·88	0·29	2·42	0·70

*Abbreviations are given in the Experimental section.

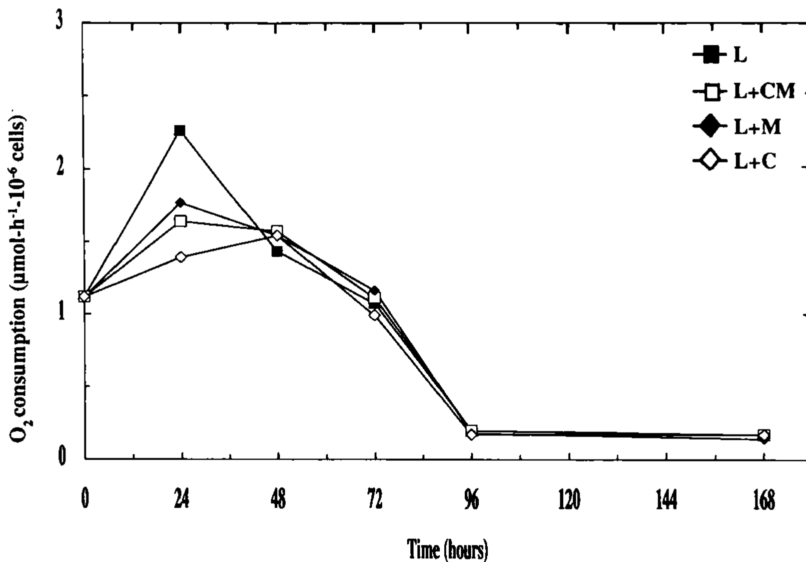


Figure 4. Cell respiration as a function of time for the control (L), L + M, L + C, and L + CM systems

The levels of total nucleotides, Table 2, were comparable in the L and the L + CM cultures (2·4–2·5 nmol per 10^6 cells), while they were 10 per cent less in L + C and 40 per cent in L + M. As expected in physiologically active cells, the AMP levels were low (representing 5 to 12 per cent of the total AMP + ADP + ATP). The ratio ATP/ADP was 0·93 in the control L cells, but reached 1·04 in the L + M and 1·11 in L + C and the remarkable value of 1·42 in cells grown in L + CM medium. The energy charges (E.C.), which ranged from 0·69 to 0·71, could be considered as constant: E.C. = ½ × (2[ATP] + [ADP])/([ATP] + [ADP] + [AMP])

Effect of aeration or carbonation on nucleotide pools and energy charges

The effects of bubbling either compressed air or CO_2 for 2 min on the cell energetics are presented in Figure 5. In the cells originating from the four types of medium (corresponding to the data detailed in Table 2), oxygenation decreased the levels of total nucleotides, mainly by decreasing the ADP pools. The ATP pools were either unchanged or only very slightly diminished. The smallest changes provoked by aeration occurred in the cells originating from the L + C medium; CO_2 treatment depressed both ATP and ADP pools. Two minutes of anoxia did not affect the L + M cells, but it increased the total nucleotides of L by 20 per cent and those of L + CM by 45 per cent; in both cases the increases were due to the formation of ATP and ADP.

The 2-min aeration or CO_2 treatments did not affect the energy charges;

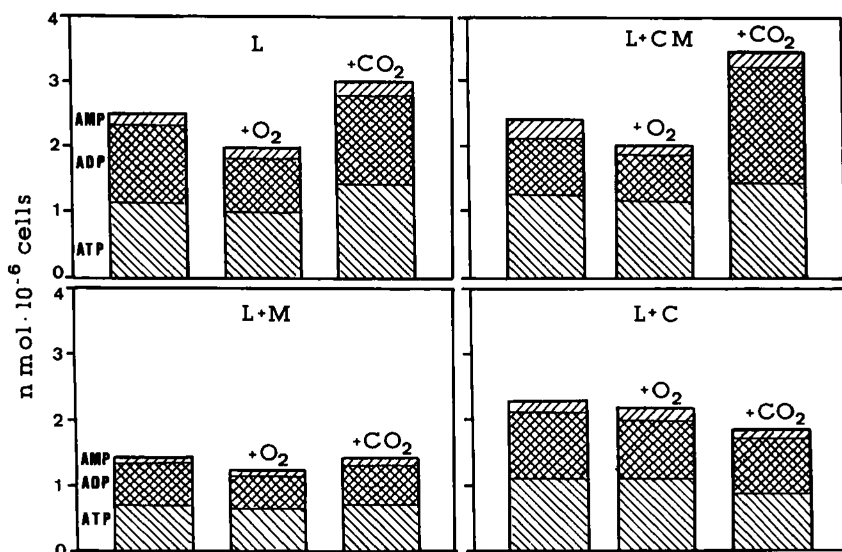


Figure 5. Effect of aeration or carbonation (fluxes of 10 ml min^{-1} for 2 min), on the nucleotide pools in the control (L), L + M, L + C, and L + CM *Euglena* cell systems. ATP, ADP, and AMP

these generally remained within the range 0·69–0·71. There was one exception; with the aerated L + CM cells, the E.C. increased significantly ($p = 0{\cdot}96$) reaching a value of 0·76 ± 0·05. The addition of CM to the lactate medium seemed to enhance the characteristics of L cells grown on lactate alone.

In vivo synthesis of ATP in the presence of added ADP and PO_4^{-3}: Effects of oxygenation or carbonation

Using suspensions of cells, rendered permeable by an extractor to the 54×10^{-8} M ADP and 27×10^{-6}M PO_4^{-3}, we measured the ATP produced during the 30 s following the injection of luciferine-luciferase preparation.

The immediate changes in ATP production, provoked by the 2-min aeration or CO_2 treatment of the four media, are shown in Table 3. In all cases, as expected, aeration increased the ATP production. This increase was much greater in L + CM cells (+173 per cent) than in the L + C cells (+80 per cent) or in the L or L + M cells (+50 per cent). Carbonation decreased the ATP production only slightly in L + M and L + CM cells, but by 25 per cent in L + C cells and by half with the L cells. Such results suggested that the cells originating from the L + CM medium are essentially immune to anoxia.

Effects of extended carbonation

The L, L + CM or L medium supplemented with CM for only 30 min prior to the experiment (L + cm) were carbonated for 20 min. While the initial capacities of the cells to produce ATP were different in L and L + CM (see Table 3), 20 min of anoxia reduced these capacities to a common level of 8 pmol 10^{-6} cells in the three types of cells examined. Aeration was then started; aliquots were sampled every minute and the extemporaneous ATP production determined. The results, presented in Figure 6, indicate that the cells orginating from the L + CM medium reacted immediately to the aeration with ATP production increasing from 7 to a plateau of 26 pmol 10^{-6} cells in 10 min. ATP production by the control cells (L medium) was not affected during the first 4 min of aeration; but the rate almost doubled between 6 and 12 min; it remained steady for a further 4 min before decreasing and stabilizing around 10 pmol 10^{-6} after 20 min. The cells originating from the L + cm, i.e. with CM present for only 30 min, rather than 24 h for the cells in the L + CM medium, exhibited intermediate characteristics: as was the case with the cells from L + CM medium, the L + cm cells reacted immediately to aeration, the ATP production doubling in the 5 first min; however, as with the control cells, the level stabilized at about 13 pmol per 10^{-6} cells, i.e. half the level attained with the cells from L + CM medium.

CONCLUSION

Addition of CM complex, corresponding to a total carbon substrate increase of 15 per cent (6·6 per cent due to malate and 8·4 per cent due to citrulline),

Table 3. Effects of aeration or carbonation (fluxes of 10 ml min^{-1} for 2 min), on ATP production, in the absence of exogeneous substrate but in the presence of excesses of ADP and phosphate

Cell system*	Initial ATP (pmol 10^{-6} cells)	Effect of air (as % of initial ATP)	Effect of CO_2 (as % of initial ATP)
L	15	+53	−46
L+M	12	+50	−7
L+C	10	+80	+24
L+CM	25	+173	−14

*Abbreviations are given in the Experimental section.

did not modify drastically the growth characteristics (Figure 1(a)) of the cells in spite of important metabolic modifications (Figure 1(b), (c), and (d) and Figure 2). Only the latency periods (lag times) and the rates of generation times were affected, when either of the two components was added alone. The sequence of utilization of the different substrates studied in this paper has been established:

1. When present, malate and citrulline were consumed at first (maximal con-

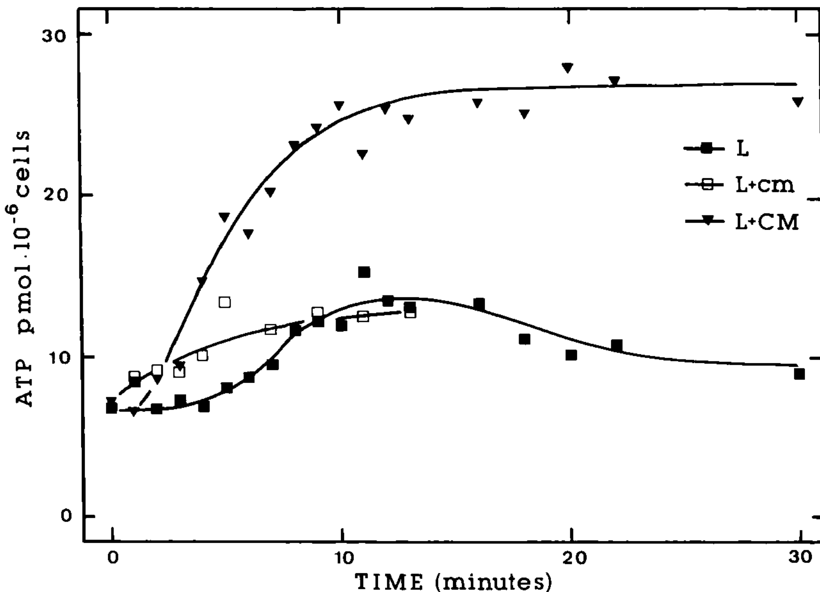


Figure 6. Effect of carbonation for 20 min, then aeration (fluxes of 10 ml min^{-1}) on ATP production in the *Euglena* cell systems L and L + CM, and in the L medium with citrulline-malate (1.5 mM) just before starting the CO_2 treatment, L + cm

sumption around 40 h) and even more rapidly (between 24 and 30 h) when added as the complex (Figure 1(d)).

2. The maximal rate of consumption of lactate occurred at about 44 h with all media except L + M, in which a delay of 8 h was observed (Figure 1(b)).
3. The rate of disappearance of ammonium ion began almost instantaneously, and reached a maximum between 30 and 36 h, in L + M and L + CM; the maximum rate occurred 24 h later in L and L + C media and was particularly intense in the L + C system (Figure 1(c)).

When the cells were grown with lactate alone, the course of lactate consumption (Figure 1(b)) coincided more or less with that for ammonium (Figure 1(c)), the maximum rate occurring, in both cases, after 45 h, and with the excretion of urea (Figure 3). This suggested a very rapid turnover of the cell proteins, peaking at 10 h, with elimination of catabolism products as urea.

Addition of the citrulline-malate complex to the medium increased the rates of lactate and ammonium consumption: these effects might be attributed to the citrulline, in the case of lactate metabolism and to malate in the case of ammonium consumption (Figures 1(b) and (c)), since amino acids are formed by amination of α-ketoacids. This view is supported by the observations that the maximum rates of the lactate utilization were reached at about the same time for the L + CM and L + C media (Figure 1(b)), while the ammonium disappearance peaked at comparable times in the media containing malate, L + M and L + CM. The retarding effect of citrulline on the ammonium consumption (Figure 1(c)) was probably due to the capacity of the cells to metabolize preferentially the three amino groups of citrulline for intense protein turnover (Figure 2(a)). The observed reinforcement of urea excretion after supplementation of the system with citrulline (Figure 3) would be expected.[30] This could also explain the weaker reabsorption of urea from L + C than from L; this is demonstrated in Figure 3, where the rates represent the net effect of the excretion and absorption processes. On the other hand, when citrulline and malate were added together as the complex, the rates of metabolism of each of the components were enhanced (Figure 1(d)). These observations indicated an overall acceleration of the consumption of the main components of the medium by the cells (Figures 1(b), (c), and (d)), but no changes in the rates of synthesis of paramylum reserves (Figure 2(c)) and total lipids (Figure 2(b)).

A very immediate and beneficial effect of the complex was evidenced. Firstly, both components of the complex were immediately consumed at initial rates which were three times greater than those characterizing the consumption of the two components considered separately (Figure 1(d)). Secondly, an intensification of the protein synthesis (Figure 2(a)) was accompanied by a reduction in the rate of urea excretion—subsequently a slight absorption of urea followed (Figure 3). This beneficial effect of the complex was attributable to the decrease, by one-third, of the C/N balance in the medium. As a matter of fact, the

addition of either M, or C, or CM, enriched the carbon content of the basic L medium by only 6 to 15 per cent; while the nitrogen content was enriched by 63 per cent with the addition of CM.

The transience of the intermediary metabolites modifies the cell energetics. The total nucleotide pools in the different culture media in environments either enriched in oxygen (by aeration) or in CO_2 were determined. Relative to the control L cells, the system with added malate, L + M, depleted the nucleotide pools (Table 2 and Figure 5) to a much greater degree but was less sensitive to O_2 (Table 3 and Figure 5) than the control L cells. The effect of citrulline alone, L + C, was less drastic but it did deplete the nucleotide levels slightly (see Table 2 and Figure 5). The complex in L + CM induced a resistance of the cells to high CO_2 concentration, characterized by a higher amount of total nucleotides with an unchanged E.C. (Figure 5 and Table 2). When these cells were progressively aerated, after having been submitted to a severe anoxia (20 min CO_2 bubbling), they displayed very quick and intense capability for synthesizing ATP in the presence of exogeneous ADP and phosphate (Table 3 and Figure 6). The nucleotide pools were always depleted by aeration (Figure 5); this is probably due mainly to ADP decreases, resulting from a transitory accelerated synthesis of nucleic acids and/or of coenzymes, which, in turn, would consume ATP molecules more rapidly than they were generated. The high levels of nucleotides found in anaerobic cell systems could be indicative of a reduction in the rates of generation of nucleic acids and coenzymes.

It has been shown[22] previously that the CM complex enhanced by carboxylation processes (transformation of either PEP in OAA or of acetyl-CoA in malonyl-CoA), without accompanying accumulation of paramylum. These carboxylations could provoke an accumulation of intermediary metabolites, able to immediately enter the TCA cycle during a subsequent aeration. The rate of the TCA cycle could thus be assessed by measuring the capacities of the cells to produce ATP in the presence of exogeneous ADP and phosphate but in the absence of added substrate. Addition of the CM complex conferred on the cells the ability to produce very quickly and, ultimately, twice as much of ATP (Figure 6) as the L system. The addition of CM just before the carbonation (L + cm on Figure 6) eliminated the lag period and, thereby, shortened the time needed to attain the ATP level characteristic of the control, L, cells. Regardless of the test conditions, the E.C. was unchanged, indicating that the normal physiological state of the cells was maintained (Table 2).

This study indicated strong analogies between the microbial model and rat hepatocytes as a means of assessing the effects of drugs on lactate metabolism and gluconeogenesis. In both systems: (1) the addition of amino acids provoked an intensification of urea excretion and (2) anoxia diminished the ATP pool.[31] Consequently, it seems probable that a CM treatment of hepatocytes would have effects comparable to those on *Euglena*: (1) reduction in the ability of the cells to excrete urea and (2) resistance to anoxia by augmenting the ATP pool. This study clearly demonstrated that the two compounds in the CM

complex have a synergistic effect on carbon and nitrogen metabolism: the malate component being clearly involved in the consumption of ammonia, while the citrulline influenced the consumption of lactate. The physical anti-asthenic effects of the CM on human subjects could then be due, on the one hand, to augmenting the detoxifying capacity of the hepathic cells with respect to the removal of ammonium ions and lactate from the blood; and, on the other, to the remarkable energetics that the CM conferred on the cells in conditions of anoxia, and even more during subsequent re-oxygenation. Physical and biochemical tests on human subjects gave results which were in accord with those reported here: after treatment with CM, lactate concentrations were reduced[12] and the rate of elimination of ammonia from the blood was increased (P. Vanuxem, personal communication).

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